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NUMBER 1

## STUDIES ON STEAM STERILIZATION OF SOILS

### II. SOME FACTORS AFFECTING MINIMUM STERILIZATION REQUIREMENTS<sup>1</sup>

BY F. J. SIMPSON<sup>2</sup> AND J. D. NEWTON<sup>3</sup>

#### Abstract

For steam sterilization of 50-gm. to 1000-gm. samples of three Alberta loam soils and a peat, the required length of treatment increased with the weight and depth of sample. The peat and black soils, high in organic matter, required longer treatments than the brown or gray soils, low in organic matter. However, identical depths of different air-dry soils were found to reach 120° C. in the same time. Moistening large samples prior to autoclaving greatly reduced the time required to reach 120° C. and to obtain sterility, but had no similar effect on small samples. A moisture content of 75% water holding capacity was optimum, reducing the 180 min. required to reach 120° C. in 6 in. of air-dry peat to 80 min. Autoclaving samples in a moist condition, however, resulted in larger increases in base exchangeable ammonia than autoclaving in an air-dry condition. Generally, minimum single treatments at 15 p.s.i. gauge produced less ammonia than at 12 p.s.i. gauge and single treatments produced less ammonia than intermittent treatments. A slight reduction in nitrate on autoclaving was observed.

#### Introduction

This investigation is part of a broad study of inter-relationships between soil microflora, soil fertility, and plant diseases. To obtain close control over the microflora, a sterile soil was desired, but one not radically changed from the original. Most investigators have used extreme treatments in their study of changes caused by heating soils, but below 200° C. the majority of the changes appear to be comparatively small (2, 8, 9, 10, 13, 16). It was therefore felt that determination of the minimum steam treatments required to achieve sterility and a measurement of the resulting alterations in the soils would be worthwhile.

Coleman, Lint, and Kopeloff (4) investigated the possibility of sterilizing soils without radical alterations, but they did not achieve sterility with their methods. They found that the application of dry heat (80° C.) to moist soil resulted in a greater destruction of bacteria and less chemical change than

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the application of various chemicals under pressure, vacuum, and in combination with heat. More recently, sterilizing soils by means of gaseous ethylene oxide has been found (6, 15) to result in a low degree of chemical change. The present investigation was confined to treating dry and moist soils in a laboratory autoclave.

### Materials and Methods

Three loam soils representing the brown, black, and gray wooded soil zones of Alberta were used for this study, in addition to a peat obtained from west of Edmonton. The soils were air-dried, large lumps were broken, and extraneous materials were removed by passing the soils through an eight mesh sieve. The coarse peat was moderately ground in an attrition type soil grinder. They were then stored in large galvanized cans.

To classify the soils as to texture, a mechanical analysis was conducted by the hydrometer method outlined by Bouyoucos (3) and the results are described in Fig. 1. The Edmonton black was the heaviest of the three loams, and

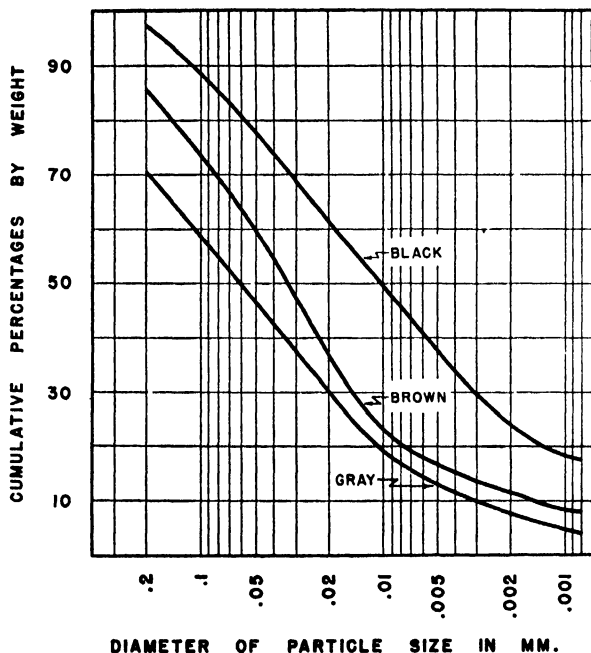


FIG 1 *Mechanical analyses of Edmonton black, Vauxhall brown, and Breton gray wooded soils.*

although the Vauxhall brown and the Breton gray were somewhat alike, the latter contained more sand and less silt. The soils were found on analysis (Table 1) to vary in fertility and organic matter content, but the pH was found to be fairly uniform.

TABLE I  
COMPOSITION OF SOILS  
(Percentage by weight, water-free basis)

Soil	Organic matter	Nitrogen	Hygroscopic moisture	Total water capacity	pH
Gray	2.8	0.16	4	54	6.2
Brown	3.5	0.20	3	57	7.0
Black	6.2	0.50	9	79	7.2
Peat	92.6	2.42	6	310	6.6

Samples of 50, 100, 400, and 1000 gm. of soil on a water-free basis were respectively treated in 300, 500, 1000, and 2000 ml. Erlenmeyer flasks. Similarly, 50, 100, and 250 gm. of peat were treated in 500, 1000, and 2000 ml. Erlenmeyer flasks. A Bramhall Deane autoclave fitted with a thermometer in the discharge line was used for treating the samples, and operated according to techniques described by Underwood (18). The flasks were placed in the autoclave on their sides to permit a more efficient removal of air from the flask and soil.

The treatments consisted of autoclaving the samples at 12 or 15 p.s.i. gauge steam pressure. The exposures were timed from the moment the desired pressure was reached. Single and daily consecutive treatments were given. The soils were treated at three different moisture levels; namely, air-dry and 50% and 100% water holding capacity. All samples except those treated in an air-dry condition were weighed and brought to 50% water holding capacity seven days prior to sterilization and incubated at room temperature. It was reasoned that this procedure would bring the soil flora to an active state and promote the germination of spores and cysts. The water content of the samples was checked immediately before autoclaving, and those to be treated at 100% water holding capacity were brought to this condition. After sterilization, the air-dry samples were moistened with sterile water. All samples were then incubated for 12 days at room temperature to allow any survivors to multiply and thus simplify detection.

Sterility was checked by plating in Petri dishes for preliminary and confirming runs. The Spray culture plate technique (17) was used to test for anaerobes. Fred and Waksman's (5) sodium caseinate agar and glucose nutrient agar were used. Sterile water was added to the flasks of soil and shaken, then 1 ml. of the mixture was transferred directly to the plate.

### Experimental

The minimum single periods of exposure to steam at 12 and 15 p.s.i. gauge pressure, required to obtain sterility, are shown in Figs. 2 and 3. The data obtained for intermittent treatments are given in Tables II and III. The

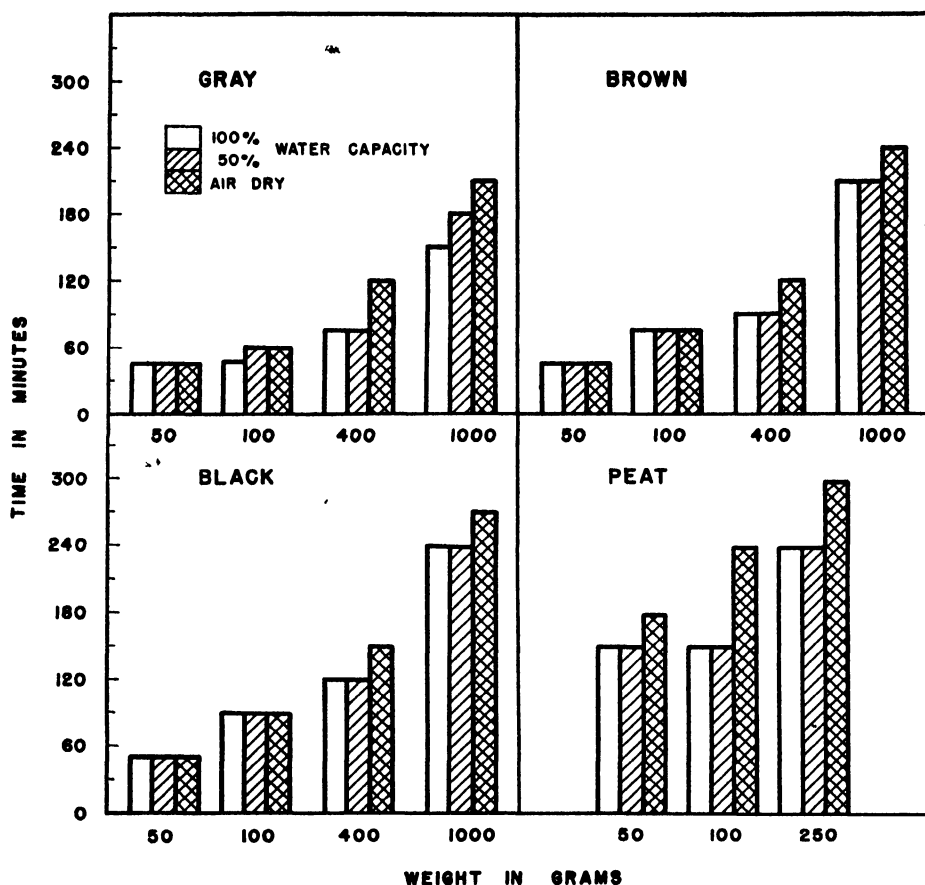


FIG. 2. Time required to steam sterilize soils in a single treatment at 12 p.s.i. gauge.

times given for intermittent treatments represent the length of each exposure required to sterilize samples when autoclaved on two successive days.

It is evident that an increase in the weight of sample with a corresponding increase in depth necessitated longer periods of exposure to obtain sterility. In addition, the different soils had different sterilization requirements. The difficulty with which different soils were sterilized appears to be correlated with organic matter content and texture. The gray loam, with the least amount of organic matter and coarsest texture, required the shortest treatment. The brown and black loams, with larger amounts of organic matter and finer textures, required longer periods of treatment. The peat, almost entirely composed of organic matter, required the severest steaming to attain sterility.

Incubating and autoclaving in a moist condition decreased the time required to sterilize large samples, but had no effect on small samples. The reduction was most evident in the peat, where it reduced the time required by as much as 30 and 90 min. In the main, moisture levels of 50 and 100% water holding capacity appeared to have an equal effect.

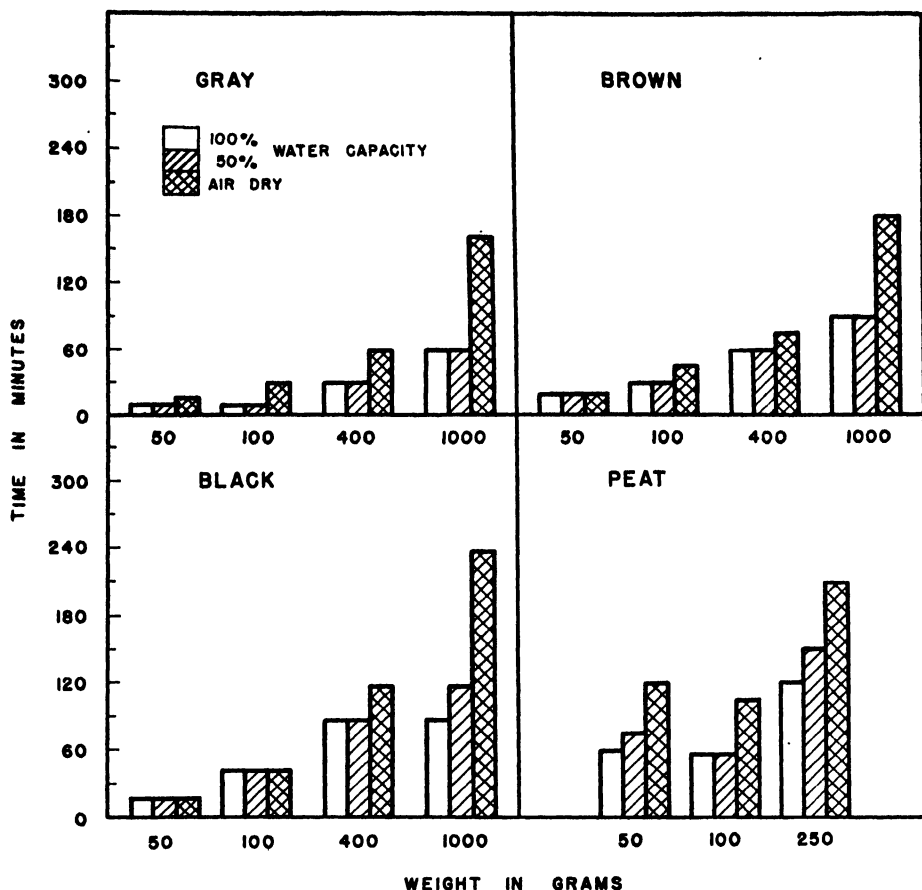


FIG. 3. Time required to steam sterilize soils in a single treatment at 15 p.s.i. gauge.

The effect of moisture may be explained by a more heat susceptible flora resulting from incubation in a moist condition prior to sterilization. However, the observation that incubation of 50- and 100-gm. samples usually did not result in a reduction of the time required to sterilize, indicates that some other factor may be of greater practical importance. The trends with respect to moisture, organic matter, and texture makes one suspect that the conductivity of the soils (14) may be the important limiting factor. If the limiting factor were steam penetration, a high moisture content would be detrimental. Water, having a specific heat about four times that of soil, would require a greater steam input to raise it to the same temperature and its presence would likely impede the penetration of steam. With small samples where the depth of soil was insignificant, steam penetration and conduction were either non-limiting or of equal efficiency. With greater depths, the presence of moisture resulted in greater efficiency than that given by steam penetration in the air-dry samples.



TABLE II

TIME REQUIRED TO STERILIZE SOILS BY STEAM AT 12 P.S.I. GAUGE IN TWO INTERMITTENT TREATMENTS

Soil	Size of sample, gm.	Length of each individual treatment, min.		
		Moisture, % water capacity		
		Air-dry	50	100
Gray	50	30	30	30
	100	45	45	30
	400	75	—	—
	1000	150	120	90
Brown	50	30	30	30
	100	60	60	60
	400	90	75	75
	1000	150	120	120
Black	50	30	30	30
	100	75	75	75
	400	90	90	90
	1000	210	120	120
Peat	50	120	90	90
	100	135	90	75
	250	240	180	180

TABLE III

TIME REQUIRED TO STERILIZE SOILS BY STEAM AT 15 P.S.I. GAUGE IN TWO INTERMITTENT TREATMENTS

Soil	Size of sample, gm.	Length of each individual treatment, min.		
		Moisture, % water capacity		
		Air-dry	50	100
Gray	50	—	—	—
	100	15	—	—
	400	15	—	—
	1000	90	60	60
Brown	50	15	15	15
	100	20	20	20
	400	60	45	45
	1000	150	—	—
Black	50	15	15	15
	100	30	30	30
	400	90	75	—
	1000	210	90	—
Peat	50	60	45	45
	100	60	30	30
	250	150	90	90

To check the effect of moisture on the sterilization process, copper-constantan thermocouples were inserted in the center of two inches of peat contained in a 2000 ml. beaker. Moisture levels of air-dry, 25, 50, 75, and 100% water capacity were studied at 15 p.s.i. gauge steam pressure. The results obtained (Table IV) indicate that moisture has a profound influence on the rate of heat penetration and that the optimum moisture content is around 75% water capacity.

TABLE IV

TIME REQUIRED FOR THE CENTER OF A 2 IN. DEPTH OF PEAT TO REACH 120° C.

(15 p.s.i. gauge steam pressure)

% water capacity	Air-dry	25	50	75	100
Time in minutes	50	30	20	15	25

The same moisture levels were used in a second test. The thermocouples were placed at one inch intervals beginning one-half inch below the surface in six inches of peat contained in a 2000 ml. beaker. Glass insulated wire was used and supported at the proper depths by threading through small holes bored in a three-eighths inch square stick. Readings were taken at two minute intervals for the first half hour, at five minute intervals during the second half hour, and at 10 min. intervals thereafter. These data are described in Fig. 4. Each gradient represents the temperatures of the soil atmosphere recorded at a specified time expressed in minutes from the moment that 15 p.s.i. gauge pressure was reached.

The gradients for the air-dry soil indicated a rapid initial rise in temperature one-half inch below the surface and one-half inch above the bottom of the beaker, but a slower and more regular rise in the center of the sample. As the steam penetrated, there was an abrupt rise in temperature with each individual point reached. By the time the steam penetrated the entire sample, the peat at the bottom had reached a fairly high temperature as a result of conduction. It required 180 min. for the peat to reach a uniform temperature of 120° C.

In moist samples, the temperatures at the extremes of the peat rose much more slowly. This would be expected because of the higher specific heat of water. The temperatures at the central points remained almost stationary until heat was conducted to them when they rose very quickly. Again, a moisture content of 75% water holding capacity appeared about optimum. With this moisture content, six inches of peat reached a uniform temperature of 120° C. in 80 min. Great difficulty was experienced in placing the thermocouples in the peat at 100% water holding capacity. The results obtained were not considered reliable and therefore are not given. Generally, 90 min. were required to raise the temperature of six inches of peat at 100% water holding capacity to 120° C.

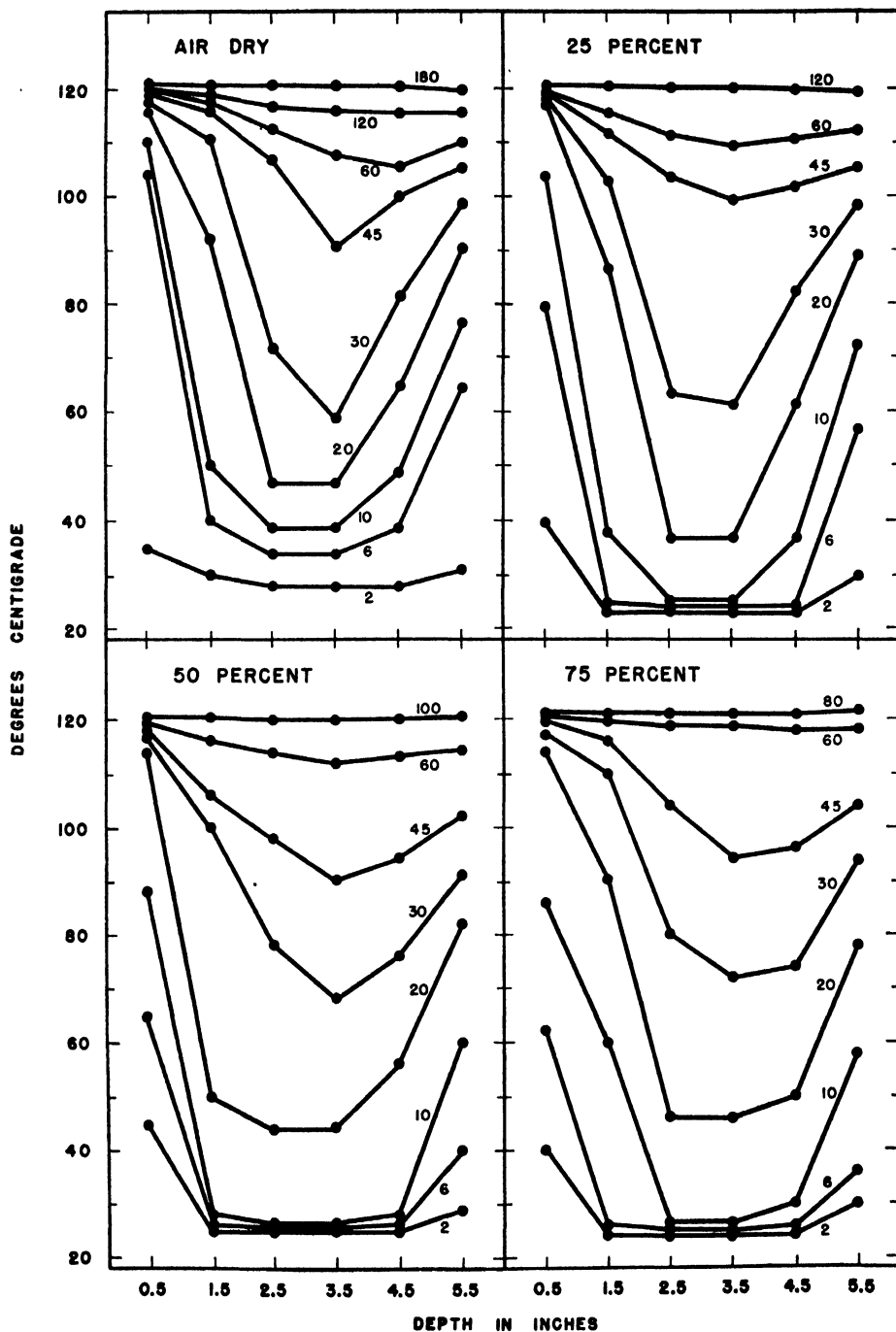


FIG. 4. Temperature gradients at various times (minutes) in six inches of peat, at different percentages of water holding capacity, treated at 15 p.s.i. gauge steam pressure.

It should be possible to remove some of the air held in the dry peat and reduce opposition to the penetration of steam by applying a vacuum of 20 in. immediately before steaming. The rate of temperature rise in the top four inches was somewhat accelerated by this procedure. However, it did not influence the time required to reach a uniform temperature of 120° C. Applying a vacuum to the moist soils had practically no effect.

The trends obtained with the peat were also found to hold for the gray and black loams. By comparison with air-dry soil a moisture content of 75% water holding capacity reduced the time required to reach a uniform temperature of 120° C. from 170 min. to 60 min. for the six inches of gray loam, and from 180 min. to 70 min. for the black loam. In sterilizing large samples of soil where depth is important, the presence of moisture is therefore desirable for efficient and rapid sterilization.

In a study of the effect of depth, thermocouples were placed in the center of air-dry samples from one to six inches deep. The results were rather surprising (Table V). For each depth, the time required to reach 120° C. was

TABLE V

EFFECT OF DEPTH ON THE TIME (MINUTES) REQUIRED TO RAISE THE TEMPERATURE TO 120° C.

(Air-dry soil at 15 p.s.i gauge steam pressure)

Depth of sample, in.	Depth of thermocouple, in.	Gray	Brown	Black	Peat
1	0.5	20	25	25	20
2	1	50	60	60	60
3	1.5	90	90	90	90
4	2	120	110	120	120
5	2.5	150	150	150	150
6	3	170	180	180	180

essentially the same. This observation was confirmed when mixtures of sand and 2 to 64% peat in geometric progression were studied. Regardless of the amount of peat in the mixture the center of a 2 in. depth reached a temperature of 120° C. in 50 to 60 min. One would therefore expect sterilization requirements of identical depths to be quite uniform.

In the original test the soils had been measured into various flasks on the basis of weight and it was realized that the specific gravity of soils would vary according to their texture and organic matter content. Therefore sterilization requirements of the air-dry soils were checked using depths of  $\frac{1}{2}$ , 1, and 2 in. A dried suspension of *Bacillus subtilis* spores was thoroughly mixed with the soils and the various depths measured into one liter Erlenmeyer flasks. These were treated in an upright position at 15 p.s.i. gauge steam pressure. Sterility

was determined by adding sterile glucose nutrient broth and examining smears after three days' incubation. Samples found to be sterile were incubated an additional three days before checking with a second smear.

These data (Table VI) indicate that the original relationship between the different soils still stands, but to a lesser degree. Although the rate of steam penetration was uniform with different soils, the organic matter and texture

TABLE VI

TIME (MINUTES) REQUIRED TO STERILIZE DIFFERENT DEPTHS (AIR-DRY SOIL AT 15 P.S.I. GAUGE STEAM PRESSURE)

Depth, in.	Gray	Brown	Black	Peat
0.5	95	105	105	120
1	105	120	120	150
2	150	180	225	240

probably modify the rate of penetration of heat in the soil atmosphere to the center of the soil aggregates. If so, one would expect the size of soil aggregates to have a measureable effect on sterilization requirements of the same and different soils. The size of aggregates in the soils studied did not appear to vary greatly, but there was a trend of increasing size from gray to black. It is also interesting to note that the colloid content of the soils (Fig. 1) increases from gray to black and to peat, or in the same order as difficulty of sterilization. These colloids and the organic matter may have a protective effect on the soil organisms. Conduction appears to have a limiting role not only in moist samples but also in dry samples.

### Degree of Chemical Change Resulting from Minimum Steam Sterilization Treatments

The effect of moisture, pressure, and single and intermittent treatments on the degree of chemical change resulting from sterilizing 50 and 100 gm. samples for their respective minimum periods was investigated. A review of the literature concerning chemical changes induced by steaming soils indicated that production of ammonia, increase in water soluble phosphorus, and destruction of nitrates occurred (9, 13, 16). The production of ammonia has been especially noted by a number of investigators. A relatively rapid and accurate method for determining base-exchangeable ammonia (12) was selected as a means of estimating the degree of chemical change induced by various minimum sterilization treatments. In addition, some nitrate determinations were made on 1:5 water extracts by the phenol-disulphonic method modified by Harper (7). All determinations were made on duplicate samples from duplicate runs. The samples were analyzed immediately after sterilizing and cooling, simultaneously with duplicate controls.

Tables VII and VIII show that the increase in ammonia varies with the organic matter content. In addition, a higher moisture content favors larger increases in ammonia. Where, however, treating the soils at 100% water

TABLE VII

INCREASES IN BASE-EXCHANGEABLE SOIL AMMONIA INDUCED BY STEAM STERILIZATION  
(50-gm. samples subjected to minimum treatments)

Treatment	Steam pressure, p.s.i. gauge	Soil moisture, % water capacity	Ammonia increases, p.p.m.			
			Gray	Brown	Black	Peat
One single	12	Air-dry	1.1	7.4	7.3	40.9
		50	5.0	12.8	11.8	43.0
		100	4.8	11.8	13.0	34.9
	15	Air-dry	1.0	4.8	4.8	21.6
		50	4.4	4.7	9.8	108.0
		100	4.8	4.8	9.9	98.1
Two intermittent	12	Air-dry	2.9	7.4	7.5	49.2
		50	2.2	12.6	14.9	115.4
		100	3.3	13.2	13.3	81.0
	15	Air-dry	—	2.0	6.4	19.0
		50	—	1.7	12.7	42.0
		100	—	2.8	12.9	38.6

TABLE VIII

INCREASES IN BASE-EXCHANGEABLE SOIL AMMONIA INDUCED BY STEAM STERILIZATION  
(100-gm. samples subjected to minimum treatments)

Treatment	Steam pressure, p.s.i. gauge	Soil moisture, % water capacity	Ammonia increases, p.p.m.		
			Gray	Brown	Black
One single	12	Air-dry	2.6	1.0	10.8
		50	5.0	3.7	15.0
		100	3.0	4.1	11.9
	15	Air-dry	2.2	2.3	6.2
		50	8.7	4.1	14.0
		100	5.6	5.7	14.8
Two intermittent	12	Air-dry	6.3	1.3	10.3
		50	9.4	8.8	20.1
		100	6.5	9.1	19.8
	15	Air-dry	1.0	2.0	6.0
		50	—	5.9	8.6
		100	—	6.4	10.9

capacity so increased the sterilization efficiency as to allow a large reduction in length of treatment, less ammonia was obtained. It is interesting to note that sterilizing the soils in an air-dry condition regardless of the longer treatments required, resulted in production of less ammonia than sterilizing in a moist condition.

Minimum treatments at a steam pressure of 15 p.s.i. gauge resulted in ammonia increases smaller than or equal to those produced at 12 p.s.i. gauge (Table IX). Somewhat larger increases in ammonia were generally obtained with two intermittent treatments than with single treatments.

TABLE IX

EFFECT OF MOISTURE CONTENT AND NUMBER OF CONSECUTIVE TREATMENTS ON THE AMMONIA PRODUCED BY STEAM STERILIZATION OF THE MINERAL SOILS

Moisture, % water capacity	Number of intermittent treatments	Average ammonia increases, p.p.m.	
		12 p.s.i. gauge	15 p.s.i. gauge
Air-dry	1	5.0	3.7
	2	5.9	4.0
	3	6.7	7.8
50	1	8.8	7.3
	2	11.3	7.3
	3	11.1	—
100	1	8.0	7.6
	2	11.0	8.2
	3	13.8	—

No definite trend of increases or decreases in the nitrate-nitrogen contents of the sterilized soils were obtained (Table X) although a small loss was observed with those treated at 15 p.s.i. gauge.

TABLE X

WATER SOLUBLE NITRATE NITROGEN IN 50-GM. SAMPLES OF STERILIZED AND NONSTERILIZED SOILS

Steam pressure, p.s.i. gauge	Moisture, % water capacity	Nitrate nitrogen, p.p.m.					
		Gray		Brown		Black	
		Control	Sterilized	Control	Sterilized	Control	Sterilized
12	Air-dry	3.0	3.1	22.4	23.1	32.0	34.4
	50	3.0	3.1	22.0	18.6	42.4	38.7
	100	3.2	2.7	23.2	18.9	42.3	38.6
15	Air-dry	3.4	3.6	21.3	20.6	32.0	31.8
	50	3.7	4.0	22.3	20.0	32.8	30.0
	100	4.0	3.2	22.0	18.2	35.0	34.3

With the exception of peat, the small changes in ammonia and nitrate-nitrogen contents of the soils caused by minimum sterilization treatments were not greater than normal variations found to occur in soils under natural conditions. By autoclaving soils in very shallow layers it appears possible to achieve sterility quickly and without radical alteration. Even under ideal conditions, however, steam sterilization induces small changes. Nevertheless, the use of steam sterilized soil in a study of the inter-relationships of soil micro-organisms, plant diseases, and soil fertility appears to be warranted.

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## THE METABOLISM OF SCOPOLETIN BY HEALTHY AND VIRUS INFECTED POTATO TUBERS<sup>1</sup>

BY SHIRLEY R. ANDREAE<sup>2</sup> AND W. A. ANDREAE<sup>3</sup>

### Abstract

After extraction of the chlorophyll, rolled leaves of potato plants infected with the leaf roll virus fluoresced a brilliant blue color in ultraviolet light. This blue fluorescence was not observed in leaves of healthy potato plants or plants infected with mild mosaic, rugose mosaic, or spindle tuber viruses. Neither was it found in unrolled leaves of leaf roll diseased plants. The blue fluorescent substance in rolled leaves had the same fluorescent and solubility properties as scopoletin.

Scopoletin was metabolized by healthy and diseased potato tubers with the production of an unstable blue intermediary that turned bright yellow on standing. This reaction was accelerated by hydrogen peroxide and inhibited by heating the tissue to 65° C., by cyanide, azide, hydroxylamine, and hydrogen sulphide. Healthy tubers were found to metabolize scopoletin more rapidly than leaf roll infected tubers.

### Introduction

Scopoletin, 7-hydroxy-6-methoxy-1 : 2-benzopyrone, has been identified as a normal constituent of *Scopolia japonica*, *Gelsemium sempervirens*, and a number of other plants (3). In ultraviolet light it shows a bright blue fluorescence similar to that of umbelliferone, a related coumarin.

In 1936 Best (4) working with tobacco plants infected with tomato spotted wilt virus noticed that in ultraviolet light the necrotic lesions were surrounded by blue fluorescent halos. A similar phenomenon was observed in infected potato, petunia, and *Nicotiana glutinosa* plants. Incipient lesions could be detected by fluorescent spots, the centers of which became necrotic within a few days. As the disease progressed the necrotic lesions and their halos spread outwards until they reached another lesion or a vein. The fluorescence then travelled along a lateral vein to the midrib and thence to the petiole and roots. This fluorescence appeared to be associated with necrosis as it was not seen in plants where infection with tomato spotted wilt did not result in necrotic lesions, e.g. in nasturtium plants infected with a mild strain of the virus. Primary lesions of tobacco mosaic virus on *Nicotiana glutinosa* plants showed relatively faint fluorescent halos; these were much less intense than those caused by the spotted wilt virus.

<sup>1</sup> Manuscript received December 15, 1948.

Contribution from the Department of Biology, University of New Brunswick, Fredericton, N.B., with financial assistance from the National Research Council of Canada.

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In 1944 Best (5) isolated the fluorescent substance from tobacco plants infected with tomato spotted wilt and showed that this substance was scopoletin. He also isolated it from healthy tobacco plants where it occurred in very small amounts, chiefly in the endodermis (4, 6).

It had been reported in the literature (1, 9, 12) that leaf roll virus infected potato tubers that showed phloem necrosis\* also showed, when viewed in ultraviolet light, a bright blue fluorescence in the necrotic areas. In 1948 the substance causing this fluorescence was isolated by Andreae (2) and it also was found to be scopoletin. It was not detected in healthy tubers or in infected tubers that did not show phloem necrosis.

The cause of the abnormal fluorescence on virus infection has not been determined. The fact that mechanical injury to tobacco plants resulted in localized patches of slightly enhanced fluorescence suggested to Best (4) that the reaction was not specific for virus infection but had a wider significance in connection with injury to the tissues. Best considered that in the healthy plant scopoletin might be concerned with growth, as topping favored the accumulation of this substance in the roots (4, 5). Theories as to how scopoletin could be produced by the plant are discussed by Best (6) in a recent paper.

Our present work has been limited to a study of conditions that result in the appearance of scopoletin in potato plants and to its metabolism by healthy and leaf roll virus infected tubers.

## Results

### *A. On the Occurrence of Scopoletin in Potato Plants*

Although the fluorescence associated with phloem necrosis in leaf roll diseased tubers had been observed previously, there were no reports concerning any abnormal fluorescence in the leaves of the infected potato plants. The fluorescence of leaves of healthy and of leaf roll diseased potato plants (varieties Green Mountain, Katahdin, Chippewa, Sebago, and Houma) was, therefore, investigated. At the same time Green Mountain potato plants infected with mild mosaic, rugose mosaic, and spindle tuber viruses were also examined for fluorescence. All plant material was obtained from the experimental plots of the Laboratory of Plant Pathology, Fredericton, N.B. The diseased plants were grown from infected tubers and were six to eight weeks old at the time of study. Some thousands of leaves were examined in the course of time. In ultraviolet light the only fluorescence visible in all healthy and infected leaves was the reddish one characteristic of chlorophyll. When, however, the chlorophyll was removed by immersing the leaves in boiling water and then in hot alcohol for a few minutes, the rolled leaves of the leaf roll

\* *Phloem necrosis is found only in tubers with primary leaf roll infection i.e. an infection contracted during the current growing season. It occurs only in certain potato varieties (8) and its appearance is influenced by storage conditions (7). Phloem necrosis in the aerial parts is constantly associated with both primary and secondary infections.*

infected plants always were found to fluoresce a brilliant blue color in ultra-violet light. Strongly rolled leaves showed a fluorescence over the entire blade, while slightly rolled leaves fluoresced generally only at their tips. This fluorescence was never observed in healthy leaves or in leaves infected with either mild mosaic, rugose mosaic, or spindle tuber viruses. Nor was it seen in unrolled leaves of leaf roll infected plants.

An attempt was made to see whether fluorescence also developed in the leaves of plants with primary leaf roll. For this purpose leaf roll infected aphids were transferred to healthy Green Mountain plants and allowed to feed on them for five days. After several weeks a slight rolling of the upper leaves of some of the exposed plants became noticeable. These upper leaves also showed a definite starch accumulation and a brilliant blue fluorescence, while the lower, unrolled leaves of the same plants showed neither.

Twenty-five years ago, Murphy (10) showed that an excess of starch in the rolled leaves is a constant feature of potato leaf roll and he concluded from his studies that the rolling of the leaves resulted from the abnormal starch accumulation. Since it was found that fluorescence in the diseased leaves appeared to be associated with rolling, it seemed likely that there might be some relationship between the abnormal starch content and the fluorescence of leaves. Examination of healthy and of diseased, rolled leaves gathered early in the morning bore out this supposition; healthy leaves showed neither starch nor fluorescence, while rolled, infected leaves showed both, and further, the starch appeared only in the portion of the blade that fluoresced. In almost every case, the areas of starch accumulation and fluorescence coincided exactly.

The blue fluorescent substance was isolated from the leaves by the same method that was used for the extraction of scopoletin from tubers. This leaf substance showed the same solubility and fluorescent properties as scopoletin.

### *B. On the Metabolism of Scopoletin by Potato Tubers*

When a slice of healthy or leaf roll diseased tuber was allowed to stand in a 10 mgm. % solution of scopoletin at room temperature, some of the scopoletin was found to be taken up by the tissue so that it became fluorescent, but in time there was a loss of fluorescence from both tissue and solution, indicating that scopoletin was being destroyed. Accompanying the loss of fluorescence were certain color changes on the surface of the tissue. The first color change consisted of the appearance of dark blue spots after the tissue had been in the solution for about four hours. These generally occurred first in the vascular regions but ultimately the whole surface became discolored. After about a day the dark blue spots turned yellow.

The addition of a drop of hydrogen peroxide to the scopoletin solution greatly accelerated the rate of loss of fluorescence and the color changes, the whole surface of the tissue turning a uniform dark blue color within a few minutes. The blue substance diffused into the solution so that both tissue and solution became colored. After the development of the blue color was

complete, the tissue and solution turned to a greenish hue and finally to a bright yellow color. The whole process required about three hours for completion. Identical color reactions occurred when scopoletin and hydrogen peroxide were added to potato juice, but they took place even more rapidly and were completed within an hour at room temperature. Potato juice to which only hydrogen peroxide or scopoletin was added showed no similar color changes over a period of 30 hr., indicating that both substances are required for the reaction with juice. Scopoletin is quite stable at room temperature for this length of time, provided it is not exposed to bright light. The juice was prepared by pulping two small potatoes in 100 ml. of water in a Waring blender and then filtering off the pulp. The juice was not kept for more than two hours. The addition of 2 ml. of a 10 mgm. % solution of scopoletin and one drop of 3% hydrogen peroxide to 2 ml. of the juice caused the rapid development of the blue color, which then gradually changed to yellow. The formation of the blue color was completely inhibited by heating the juice to 65° C. for 15 min., by sodium cyanide (0.001 *M*), sodium azide (0.001 *M*), sodium sulphite (0.01 *M*), hydroxylamine (0.01 *M*), ascorbic acid (75 mgm. %), very small amounts of hydrogen sulphide, and sodium fluoride (1 *M*). Sodium fluoride in a concentration of 0.01 *M*, however, had no appreciable effect on the reaction. The blue color, once formed, could be turned yellow immediately by heating, or by the addition of cyanide, sodium sulphite, ascorbic acid, alkali, or alcohol. No means was found of inhibiting the change from blue to yellow.

The yellow substance appeared to be quite stable at room temperature and was isolated by the following procedure. One hundred milligrams of scopoletin was dissolved in about 5 ml. of hot alcohol and poured into 500 ml. of potato juice. The addition of 2 ml. of 3% hydrogen peroxide turned the solution an inky blue color. On standing at 38° C. for about 15 min. the solution became greenish-yellow. Two milliliters more of hydrogen peroxide was then added upon which the color again became an inky blue. This procedure was repeated until the addition of hydrogen peroxide to the greenish-yellow solution did not cause further appearance of the blue color. The juice was then allowed to stand until the color changed from greenish-yellow to yellow. On adjusting the pH to 4, a bulky yellowish precipitate settled out. This was centrifuged down and the colorless supernatant fluid discarded. After being dissolved in alkaline water (pH 8) and reprecipitated at pH 4, the yellow substance was separated from the bulky precipitate by being shaken with 200 ml. of alkaline alcohol (3 ml. of concentrated ammonium hydroxide per liter of alcohol). This extraction was repeated five times. The alcohol extract was concentrated to about 500 ml. under reduced pressure and passed through an alumina adsorption column. The yellow substance was strongly adsorbed at the top of the column. The column was rinsed with 200 ml. of alcohol and the yellow substance eluted by an alkaline solution (pH 10) of five parts of alcohol to one part of water. The 300 ml. of eluate were concentrated to 20 ml. under reduced pressure. On adjusting the concentrate to pH 4, the

substance settled out in a pale yellow, noncrystalline state. In alkaline solution, this substance was bright yellow and showed a brilliant yellow fluorescence in ultraviolet light. Work is in progress on its identification.

Preliminary studies indicated that the fluorescence of scopoletin solutions was destroyed more rapidly by healthy tuber tissue than by leaf roll infected tissue. Two slightly different procedures were employed to study this effect. By Method A, duplicate cylinders 1 cm. in length and 6 mm. in diameter were punched out of the heel end of a Green Mountain tuber. After being rinsed momentarily in water, the duplicate cylinders were placed in a large culture tube containing 5 ml. of a 0.075 mgm. % solution of scopoletin. One-half milliliter of a 4% solution of sodium fluoride was added to the tube to inhibit bacterial growth. The cylinders were incubated in the scopoletin-fluoride solution at 38° C. for 24 hr. They were then removed from the solution, rinsed, placed in small individual tubes, and the intensity of their fluorescence in ultraviolet light was estimated visually. The intensity of the fluorescence of the scopoletin solution in which the cylinders had been incubated was also estimated. By this method cylinders from 36 tubers and the solutions in which they had been incubated were separated on the basis of their relative fluorescence into three groups viz. strongly, moderately, and slightly fluorescent. An eye of each tuber studied was planted and the plants were inspected for symptoms of leaf roll after eight weeks of growth. The classification of the tubers as healthy or diseased was based on these indexing results.

Table I shows the number of cylinders from the 36 tubers with a strong, moderate, or slight fluorescence after incubation in a 0.075 mgm. % solution, of scopoletin for 24 hr., and the relation of the intensity of the fluorescence to

TABLE I

	Fluorescence of cylinders*		
	Strong	Moderate	Slight
No. of healthy cylinders	—	3	17
No. of diseased cylinders	12	1	3

\* The results are given for only one cylinder from each tuber as duplicate cylinders showed the same fluorescence.

healthy and leaf roll virus diseased tissue. Table II shows the relative fluorescence of the scopoletin solutions after incubation for 24 hr. with the above cylinders. These two tables indicate that in slightly over 80% of the tubers studied the rate of loss of fluorescence from both tissue and solution was markedly greater with healthy material than with leaf roll diseased material.

In Method B similar cylinders were taken in duplicate from both the eye and heel ends of each tuber. Duplicate cylinders were placed in 5 ml. of a 0.1 mgm. % solution of scopoletin in a large culture tube and incubated for two

TABLE II

	Fluorescence of solutions		
	Strong	Moderate	Slight
No. of solutions incubated with healthy cylinders	3	1	16
No. of solutions incubated with diseased cylinders	13	—	3

hours at 38° C. The cylinders were then removed, rinsed in water, placed in a dish with small partitions, and covered with water. They were incubated for a further two hours and then each cylinder was placed in a small test tube and the intensity of its fluorescence in ultraviolet light was estimated visually. By this method cylinders from 85 tubers were separated into three groups depending on their relative fluorescence. Each of the 85 tubers studied was indexed in the greenhouse and the classification of the tubers was based on the indexing results.

Table III shows the relative fluorescence of the cylinders after the above treatment and the relation of the intensity of the fluorescence to the healthy and diseased tissue. These results indicate that there was a markedly greater loss of fluorescence from the healthy cylinders than from the infected cylinders in 85% of the cases.

TABLE III

	Fluorescence of cylinders*		
	Strong	Moderate	Slight
No. of healthy cylinders	5	1	38
No. of diseased cylinders	35	3	3

\* The results are given for only one cylinder from each tuber as duplicate cylinders showed the same fluorescence.

In both methods duplicate cylinders gave the same readings. Cylinders from eye and heel ends also agreed. The groupings obtained by two different workers were almost always identical. The cylinders could be readily separated with a little experience; the liquids were sometimes more difficult to judge. The concentration of scopoletin used in these two methods was too low to cause any appreciable color change on the tissue surface.

### Discussion

It is not known whether scopoletin is a metabolic intermediary in healthy potato plants. The fact that it has been isolated from a number of healthy plants indicates that in some species at least it is a normal metabolic product.

In potato plants, however, it is possible that it may be produced only under certain conditions, of which but two, namely infection with the leaf roll and the tomato spotted wilt viruses, are known at the moment. As infection with mild mosaic, rugose mosaic, and spindle tuber viruses did not cause the appearance of a blue fluorescence in the leaves, this effect does not seem to be a property of viruses in general.

Whether or not scopoletin is a normal metabolite of potato plants, the results show that it can be destroyed by tuber tissue with the production of colored compounds. As the destruction of this substance by the tubers is accelerated by hydrogen peroxide and inhibited by heating the tissue to 65° C., and by low concentrations of cyanide, azide, hydroxylamine, and hydrogen sulphide, a thermolabile peroxidase would appear to be involved. It should be mentioned here that the addition of potassium permanganate to a scopoletin solution causes identical color changes. However, the yellow substance so formed differs from that produced enzymatically in that the former will turn blue again on acidification, while the latter only turns a paler yellow.

If scopoletin is a normal metabolic intermediary in potato plants, its accumulation on infection with the leaf roll virus may be simply ascribed to the decreased ability of the plant to metabolize it so that it is not removed as rapidly as it is formed. Although we do not know why leaf roll infection causes the decreased ability of the plant to metabolize scopoletin, there are several possibilities that at present are being investigated. First, the virus might cause an underproduction of the enzyme or enzymes involved, or the production of them in a less active form. Second, the infection might result in the elaboration of inhibitory agents. Smith and Paterson (13) and Newton (11) have reported a high concentration of ascorbic acid in leaf roll infected potatoes. As we have seen, ascorbic acid in sufficient concentration will inhibit the destruction of scopoletin by tubers. However, more data must be obtained on the actual concentration of ascorbic acid in infected tubers before it can be decided whether or not it is high enough to account for the markedly decreased rate of scopoletin metabolism.

The fact that starch and scopoletin both first accumulate in the tip of the blade suggests that the virus may invade the blade from the tip and from there spread throughout it, causing a more or less general metabolic disturbance in the invaded areas. However, there is as yet no adequate evidence that the starch accumulation is due to a disordered metabolism of this substance, and other factors, such as permeability or translocation abnormalities that could cause a starch accumulation, must not be neglected.

The question arises as to whether any of the fluorescent or metabolic properties of leaf roll infected potato plants can be utilized in testing for infection. As far as the fluorescence in the leaves is concerned, this can be dismissed as being no more useful a criterion of a diseased condition than the presence of an abnormal amount of starch, for under certain conditions of growth, neither starch accumulation nor fluorescence is found in infected plants. Plants in which starch and scopoletin accumulate usually show an



obvious rolling of the leaves and other external symptoms of leaf roll, so that there seems little point in examining their leaves for fluorescence except in doubtful cases where it is not known whether the rolling is of parasitic nature.

A more important consideration is a biochemical test for leaf roll infection in the tubers as at the moment no satisfactory one exists. Newton (11) has published a method for the diagnosis of leaf roll in tubers that is based on their ascorbic acid content, but more must be known about the specificity of such a procedure before it can be accepted. The difference in the rate of scopoletin metabolism between healthy and leaf roll infected tubers also seems to offer some promise for a biochemical test, but as yet sufficient work has not been done on tubers infected with leaf roll and with other virus diseases to determine its specificity.

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# ROW TREATMENT OF SOIL WITH TETRAMETHYLTHIURAM DISULPHIDE FOR CONTROL OF BLACKROOT OF SUGAR-BEET SEEDLINGS. I. GREENHOUSE TESTS<sup>1</sup>

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## Abstract

The prevention of blackroot of sugar-beet seedlings has been attempted in greenhouse experiments, by row applications of different proprietary compounds to naturally-infested field soils representing three distinct types and differing biologically with respect to associated pathogens.

Segmented seed, var. U.S. 215 × 216, was used in all the tests as was also a standard, commercial fertilizer. In earlier trials, the chemical under test and the fertilizer were added separately to a measured volume of rootrot soil. In later trials, the chemical mixed with the fertilizer was incorporated with the soil by a method approaching more closely that employed in field practice. The two procedures, designated as the precision and the surface application method, respectively, are described in detail. Calculations for the requisite amounts of chemical and fertilizer were made on the basis of sugar-beet rows in the field being spaced 22 in. apart, *i.e.*, 23,760 linear feet of row per acre.

Tetramethylthiuram disulphide (50%, nonwetable), when applied as Arasan or D-419 at the rate of 3 to 4 lb. per acre, consistently proved to be by far the most effective of the chemicals tested, in reducing pre- and postemergence blackroot. Arasan, which was investigated more intensively than any of the other chemicals, was found (a) to retain its fungicidal capability after being mixed for as long as 14 months with a commercial fertilizer, (b) to be effective in reducing the incidence of the disease in three quite different types of soil, and (c) to remain effective in the soil over relatively long periods of time. These beneficial effects are contingent, however, upon correct placement of the chemical, best results having been obtained when the seed germinated in, and the seedlings grew up through, soil impregnated with the Arasan-fertilizer mixture. Up to 21° C., Arasan, at a rate as high as 4 lb. per acre, will, in a moderately wet soil, effectually control the disease without injury to the seedlings. At 27° to 29° C., moderate injury to roots and lower hypocotyl resulted from a 3 lb. per acre application of the chemical and, following a 4 lb. application, root and hypocotyl injury was accentuated, typical foliar symptoms appeared, and mortality of seedlings was appreciable.

Of the other chemicals tested, 2,4,5-trichlorophenyl acetate (Mycotox No. 1), 20% copper trichlorophenate (C-119), 50% 2,4,5-trichlorophenyl chloroacetate (F-800), and a product of undisclosed chemical composition (CCH-358 or 358A) showed encouraging possibilities.

## Introduction

In southwestern Ontario, as in other sugar-beet-growing areas of the mid-continental humid belt, the disease commonly known as blackroot constitutes one of the most important limiting factors in the obtaining of adequate and uniform stands of seedlings. Several fungi, including *Pythium* spp., *Phoma Betae* (Oud.) Frank, *Rhizoctonia Solani* Kuehn, and *Aphanomyces cochlioides*

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Drechs., are known to be capable of causing the disease in Ontario.\* In a recent comprehensive review of the blackroot situation as it pertains to the humid area in the United States, Coons, Kotila, and Bockstahler (1) state that a differentiation can be made between what they term acute and chronic phases of the disease. According to these authors, the acute stage is manifested by the killing of young plants during germination or within a week or two after emergence from the soil. Seedlings do not always die but many of those that survive the initial attack never fully recover and their main or lateral roots show a diseased condition that is often characteristic of the primary pathogen involved. Thus, various disease aspects may become apparent in the later stages of growth of the plant, such aspects being regarded as manifestations of the chronic phases of blackroot.

The distinctions that have been made between the acute and the chronic phase of blackroot by the above-mentioned investigators are as applicable to the disease as it occurs in Ontario as to that prevalent in many of the sugar-beet-growing areas of the United States. Both phases have been recognized and have been under investigation by the present authors for the past several years. The acute phase, however, has received the greater amount of attention. In the acute stage, both pre- and postemergence damping-off play a role in the failure of stands. Field observations to date have indicated, however, the considerably greater destructive capacity of the postemergence phase. Season after season, a definite sequence has been observed. Just when emergence is approaching its maximum, blackroot makes its appearance. After a period of several days, during which the seedlings are especially susceptible to attack, stands in many instances are either completely destroyed or reduced to the extent that it becomes questionable whether they should be saved even though, following the period of high mortality, the surviving seedlings show evidence of 'coming out' of blackroot.

Although the terms damping-off and blackroot are used almost synonymously, nevertheless the latter is somewhat of a misnomer; for, in many infected seedlings, the roots are not attacked at all. Rather, the hypocotyl is the vulnerable part of the young plant. Thus, strictly speaking, the term 'black hypocotyl' would be more accurately descriptive of the condition of the seedling. In Europe, the term black leg (8) is used to denote the hypocotyl type of damping-off. The hypocotyl becomes infected usually at or near the soil level. Infection spreads rapidly and the hypocotyl is soon killed or reduced to a thin, black thread that is incapable of supporting the seedling in an upright position.

Because seedlings are especially susceptible to attack by parasitic organisms during a certain period in their early development and because, further, the point of attack is fairly localized, it would seem reasonable to suppose that, if the seedlings were afforded additional protection against the pathogen at the

\* *Aetiological and other phases of the blackroot problem beyond the scope of the present paper will be fully dealt with in a forthcoming publication by the second author.*

right time and place, they might have a greater chance of survival. Accordingly, during the past three years, the present authors have carefully investigated the possibility of modifying or controlling the disease by adding, to the surface layers of infested soil, various of the newer chemicals possessing fungicidal or fungistatic properties. Of the many protectants tested, formulations containing 50% tetramethylthiuram disulphide as the active agent have given the consistently best results and for this reason most of the experiments described below have centered around the use of this chemical. This new line of attack was adopted only after treatment of seed with several of the older and many of the newer proprietary materials had failed to give adequate protection to the seedling.

### Material and Methods

In experiments carried out under ordinary environmental conditions in the greenhouse, standard wooden flats  $24 \times 12 \times 4$  in. were employed. In tests requiring more precise control of environmental conditions, Wisconsin temperature tanks were used. In all experiments the soil was watered moderately as required. Many of the tests were carried out during the winter months in a heated greenhouse, where, as thermographic records show, the temperature of the air varied considerably in accordance with changing outdoor environmental conditions.

In some experiments the seed alone was treated; in others, the soil alone; and in still others, both the seed and the soil. Counts were made daily and emergence was expressed as the number of seedlings that appeared above ground in relation to the number of seeds planted. The difference between the number of seedlings that emerged and the known germinating capacity of the seed was regarded as indicating fairly closely the reduction in stand due to pre-emergence damping-off. Incidence of postemergence blackroot was determined numerically by expressing the number of typically infected seedlings as a percentage of the number that emerged from the soil. Experiments were allowed to run five weeks, at the end of which time roots of seedlings were given a final examination.

#### *Seed*

Segmented seed designated as U.S. 215  $\times$  216 was used throughout the course of the experiments. This seed, furnished by the Canada and Dominion Sugar Company, Chatham, Ont., was representative of warehouse stock that was being distributed by the company to Ontario growers. A first lot of seed obtained in 1945, and used in the 1946-47 tests, had a germinating capacity of 98%. A second lot obtained in 1947, and used in the 1948 tests, had a germinating capacity of 109%. The 1947 seed was noticeably larger than that obtained in 1945, and, in germination tests, the former was found to produce a higher proportion of 'doubles' than did the latter. To ensure as uniform germination as possible, seed to be used in a given test was examined under a dissecting microscope and off-size as well as nonviable-appearing particles

were discarded. In the flats, the seeds, spaced one-half inch apart, were always planted 44 to the row, with three to five equally-spaced rows per flat. In both flats and Wisconsin tank cannisters, the seeds were planted  $\frac{1}{2}$  to  $\frac{5}{8}$  in. deep.

### Soil

In all the preliminary and in most of the later experiments, the soil used was a Brookston clay loam obtained from fields where, after repeated failures due to blackroot (acute phase), attempts to grow sugar beets had been abandoned. Mortality of seedlings grown in this soil, even with a high-phosphate commercial fertilizer added, was always high, the postemergence phase of the disease under conditions of moderate watering causing on the average nearly 50% loss of seedlings. Repeated isolations from blackroot-infected seedlings grown in this soil yielded principally cultures of *Aphanomyces cochlioides* Drechs.

As the investigations progressed, the trials were extended to include two other quite different types of soil in which, also, repeated loss of seedlings had been sustained. One of these was a light sandy loam (hereinafter referred to as 'Exeter' sand), and the other, a dark-colored clay of the Clyde series. Blackroot-infected seedlings in the 'Exeter' sand invariably yielded cultures of *Pythium aphanidermatum* (Edson) Fitzpatrick, while from those grown in the Clyde clay, *A. cochlioides* and *Pythium ultimum* Trow were isolated in the ratio of 17 to 5, respectively.

### Fertilizer

To all flats or containers except those serving as untreated checks, a 2-16-(8, 10, or 6) commercial fertilizer was added at the rate of either 300 or 400 lb. per acre. Half of the fertilizer was placed in a band  $1\frac{1}{2}$  in. below the seed, the other half either in contact with the seed, or on the surface of the soil in which latter case it was worked into the top inch of soil before the planting of the seed.

### Protectants

Although, as previously stated, most of the investigational work herein reported centers around trials in which tetramethylthiuram disulphide was used, nevertheless, in conjunction with these experiments, a number of other materials were subjected to careful test. These proprietary compounds, provided gratuitously by the manufacturers or distributors indicated below, included the following:

*Arasan*—Tetramethylthiuram disulphide, 50% active material, nonwett-able; Canadian Industries Limited, Chatham, Ont.

*Tersan*—Tetramethylthiuram disulphide, 50% active material, wettable; Canadian Industries Limited, Chatham, Ont.

*Fermate*—Ferric dimethyldithiocarbamate, 70% active material, wettable; Canadian Industries Limited, Chatham, Ont.

*9-B*—Zinc 2,4,5-trichlorophenate; Dow Chemical Co., Midland, Mich.  
*C-119*—Copper trichlorophenate, 20%; Dow Chemical Co., Midland, Mich.  
*CCH355, 356, and 358 or 358A*, respectively; Dow Chemical Co., Midland, Mich.

*F800*—2,4,5-trichlorophenyl chloroacetate, 50% active material; Dow Chemical Co., Midland, Mich.

*Mycotox No. 1*—2,4,5-trichlorophenyl acetate; Givaudan-Delawanna Inc., New York, N.Y.

*Cupferron*—Ammonium nitrosophenylhydroxylamine; Eastman Kodak Co., Rochester, N.Y.

*D-419*—Tetramethylthiuram disulphide, 50% active material, nonwetable; Naugatuck Chemical Division, U.S. Rubber Co., Elmira, Ont.

*Dithane D14*—Disodium ethylene bisdithiocarbamate hexahydrate, 25%; P. N. Soden Co., Toronto, Ont.

*Dithane Z-78*—Zinc ethylene bisdithiocarbamate, 65%; P. N. Soden Co., Toronto, Ont.

### *Procedure*

The protectants, either alone or mixed with fertilizer, were added to the soil in two ways, which, for convenience of description, may be termed the precision and the surface methods of application.

(a) *The precision method.*—A 1-in.-deep layer of the infested field soil was spread uniformly in a flat, fertilizer was spread in the requisite number of bands, and then another inch of soil was added. Thin strips of wood,  $22 \times 1\frac{1}{4} \times \frac{1}{4}$  in., were affixed in pairs to delimit 2-in.-wide enclosures running lengthwise of the flat. Meanwhile, a quantity of sieved rootrot soil had been brought to an almost air-dry condition by exposure on a greenhouse bench. To an amount of this soil just sufficient to fill the space enclosed by the wooden frame, *i.e.*, 55 cu. in., the protectant was added at the desired rate, the soil and chemical then being shaken together in a flask. After thorough mixing, the contents of the flask were used to fill the  $22 \times 2 \times 1\frac{1}{4}$ -in. strip delimited by the wooden frames. The method has been graphically illustrated in an earlier publication (6). Calculations for the amounts of both protectant and fertilizer were made on the basis of the sugar-beet rows in the field being 22 in. apart, *i.e.*, 23,760 linear feet of row per acre. Thus, to determine the quantity of protectant required for a row application in a flat, at a rate, say, of 3 lb. per acre, it was only necessary to calculate the fraction necessary for 22 in., on the basis that in actual field practice 3 lb. would be distributed over 23,760 linear feet.

(b) *The surface application method.*—On an inch of rootrot soil in the bottom of a flat, the fertilizer was spread in bands in accordance with the number of rows of seeds to be planted. Then, two additional inches of the rootrot soil were added. Meanwhile, the amounts of the protectant and the fertilizer, determined as above for row application, were thoroughly mixed.

The mixture, having been uniformly spread over the surface of the 2-in.-wide strips where the seed was to be planted, was then worked thoroughly into the top  $1\frac{1}{4}$  in. of soil. The seeds were planted in the center of the treated strips at a depth of about  $\frac{1}{2}$  in.

## Investigational

### (a) Preliminary Tests

Of the various protectants listed above, Arasan, fortunately, was one of the first to be investigated. In the earliest exploratory tests, this compound at the rate of 200 lb. per acre was applied to naturally-infested soil by the precision method described above. Inhibition of germination and extreme toxicity to seedlings followed this rate of application. In several series of subsequent tests, the amount of Arasan was scaled down through a range of descending rates of application per acre as follows: 150, 125, 100, 75, 50, 25, 15, 10, 5, 3, and 1 lb., respectively. In all these tests a 2-16-10 commercial fertilizer was applied at the rate of 300 lb. per acre. In one series, the seed but not the soil (except for the addition of fertilizer) was treated with Arasan at the rate of  $\frac{1}{2}$  lb. of the protectant per 100 lb. of seed. In another series, untreated seed was planted in lots of the same soil, with and without the addition of fertilizer. Four flats comprised a test unit for each treatment. These various trials were carried out during January, February, and March, 1946. The results obtained are summarized in Table I.

TABLE I

EFFECT OF TREATMENT OF SEED, OF SOIL, AND OF SEED AND SOIL WITH ARASAN, ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

	Treatment		Emergence of seedlings, %*	Degree of toxicity to seedlings	Incidence of blackroot, %*	
Seed	Soil				Pre-emergence phase (approx.)**	Post-emergence phase
	Arasan	Fertilizer (2-16-10)				
½ % Arasan	15 lb.***	150/150 lb.***	80.3	Severe	17.7†	0
“	10 “	“	89.3	“	8.7†	0
“	5 “	“	84.0	“	14.0†	0
“	3 “	“	97.7	Slight	0.3	6.2
“	1 “	“	94.1	None	3.9	15.2
“	Nontreated	“	95.4	“	2.4	20.8
Nontreated	“	“	96.2	“	1.8	26.7
“	“	Nontreated	93.9	“	4.1	42.7

\* Average of four flats.

\*\* Obtained in this and succeeding tables by subtracting figures for emergence from germinating capacity of seed i.e., 98%.

\*\*\* Per acre application, the numerator-denominator designation in this and succeeding tables indicating that half the fertilizer was applied with, the other half, below the seed.

† These values represent losses due more probably to toxic effects than to parasitic attack.

As Table I indicates, no blackroot occurred when Arasan was applied at the rates of 15, 10, and 5 lb. per acre. At these concentrations, however, not only was emergence reduced but also severe toxicity symptoms were produced both underground and aboveground on the seedlings. Arasan at 3 lb. per acre had no deleterious effect on germination, induced only scarcely perceptible toxicity symptoms, and inhibited blackroot to the extent of all but 6.2%. At this rate of application, the disease did not appear until the 22nd day after planting. At the 1 lb. per acre application, blackroot developed to the extent of 15.2%, the first appearance of the disease having been noted on the seventh day after planting. When the seed alone was treated, incidence of the postemergence phase of the disease reached 20.8%, thus indicating in these, as in previous trials (7), the ineffectiveness of seed treatment. Highest incidence of blackroot, *i.e.*, 42.7%, occurred when neither seed nor soil was treated with Arasan and when, too, fertilizer was omitted. It will be noted that the addition of fertilizer to the soil reduced the incidence of the post-emergence phase of the disease by about 16%. Even in the untreated soil, the pre-emergence phase of the disease was in these tests of only slight significance.

#### *(b) Confirmatory Tests*

Following the preliminary trials described above, many confirmatory tests have been carried out in which attention was concentrated on the 3 or 4 lb. to the acre applications of Arasan. One such test was completed in May-June, 1946, under summer greenhouse conditions. In this test, the Arasan was applied at the rate of 4 lb. per acre. Another test was completed in December, 1946-January, 1947, in the same location but under winter conditions,\* the Arasan in this case being applied at the rate of 3 lb. per acre. In both of these tests, the Arasan and the commercial fertilizer were applied by the precision method and four flats comprised the test unit for each treatment and check. Details in connection with these particular trials together with results obtained are recorded in Table II.

As Table II shows, only a negligible percentage of blackroot occurred when the seed and soil, or the soil alone had been treated with Arasan. Thus, the results closely confirmed those obtained in the preliminary trials. While seed treatment was undoubtedly effective in reducing pre-emergence damping-off, nevertheless, such treatment afforded only slight protection against the postemergence phase of the disease. In the nontreated checks, the incidence of postemergence blackroot was 27.1% higher in the summer experiment than in the one completed during the winter months. This higher incidence of the disease is probably to be correlated with the consistently higher soil temperature that prevailed throughout the duration of the summer experiment. In the nontreated checks, it will be noted also that pre-emergence damping-off reduced emergence to the extent of about 22% in both the

\* Representative flats from this test were on display at the meetings of the American Society of Sugar Beet Technologists, held in Detroit, January, 1947.



TABLE II

EFFECT OF TREATMENT OF SEED AND OF SEED AND SOIL WITH ARASAN ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Time of experiment	Treatment			Emergence of seedlings, %*	Incidence of blackroot, %*	
	Seed	Soil			Pre-emergence phase (approx.)	Post-emergence phase
		Arasan	Fertilizer (2-16-10)			
May-June 1946	$\frac{1}{2}$ % Arasan	4 lb.**	200/200 lb **	96.0	2.0	1.0
	Nontreated	"	"	91.8	6.2	2.0
	$\frac{1}{2}$ % Arasan	Nontreated	"	96.3	1.7	53.8
	Nontreated	"	"	76.1	21.9	65.0
Dec. 1946-Jan. 1947	$\frac{1}{2}$ % Arasan	3 lb.	200/200 lb.	87.0	11.0	0.6
	Nontreated	Nontreated	"	75.3	22.7	37.9

\* Average of four flats.

\*\* Per acre applications.

summer and the winter tests. In Fig. 1, the difference between nontreatment of the soil and treatment with Arasan at 4 lb. per acre are clearly shown, the two series of flats illustrated being taken from the May-June, 1946 experiments.

### (c) Supplementary Tests

While the experiments described above had furnished considerable evidence as to the effectiveness of Arasan in controlling blackroot, nevertheless, many points in connection with the use of this protectant required further investigation. One of the most important of these centered around the possibility of simplification of the method of application. Up to this time, the protectant and the fertilizer had been added separately by the impractical and time-consuming precision method. If the fungicide and the fertilizer could be mixed and the mixture applied to the soil without diminution of effectiveness, an improvement would be effected. The reports of Martin (10) in 1933, of Cunningham (2) in 1936, of Cunningham and Wessels (3) in 1939, and, more recently, of Doran (4, 5), all established precedent for supposing that a fungicide-fertilizer mixture might be used with success in the present problem. If, in addition, the mixture could be applied to the soil in a manner more closely approaching that employed in actual field practice, the mechanics of control by this means would be reduced to a still more practical basis. Of importance, too, from the practical standpoint would be any information that could be gained about the protectant in regard to (a) its proper placement in the soil with reference to the seed, (b) the length of time it might remain effective in the soil, and (c) how its efficacy might be modified by soils of different type and by temperature and moisture of the soil. In addition, it was realized that a continuous effort should be maintained to find, if possible,

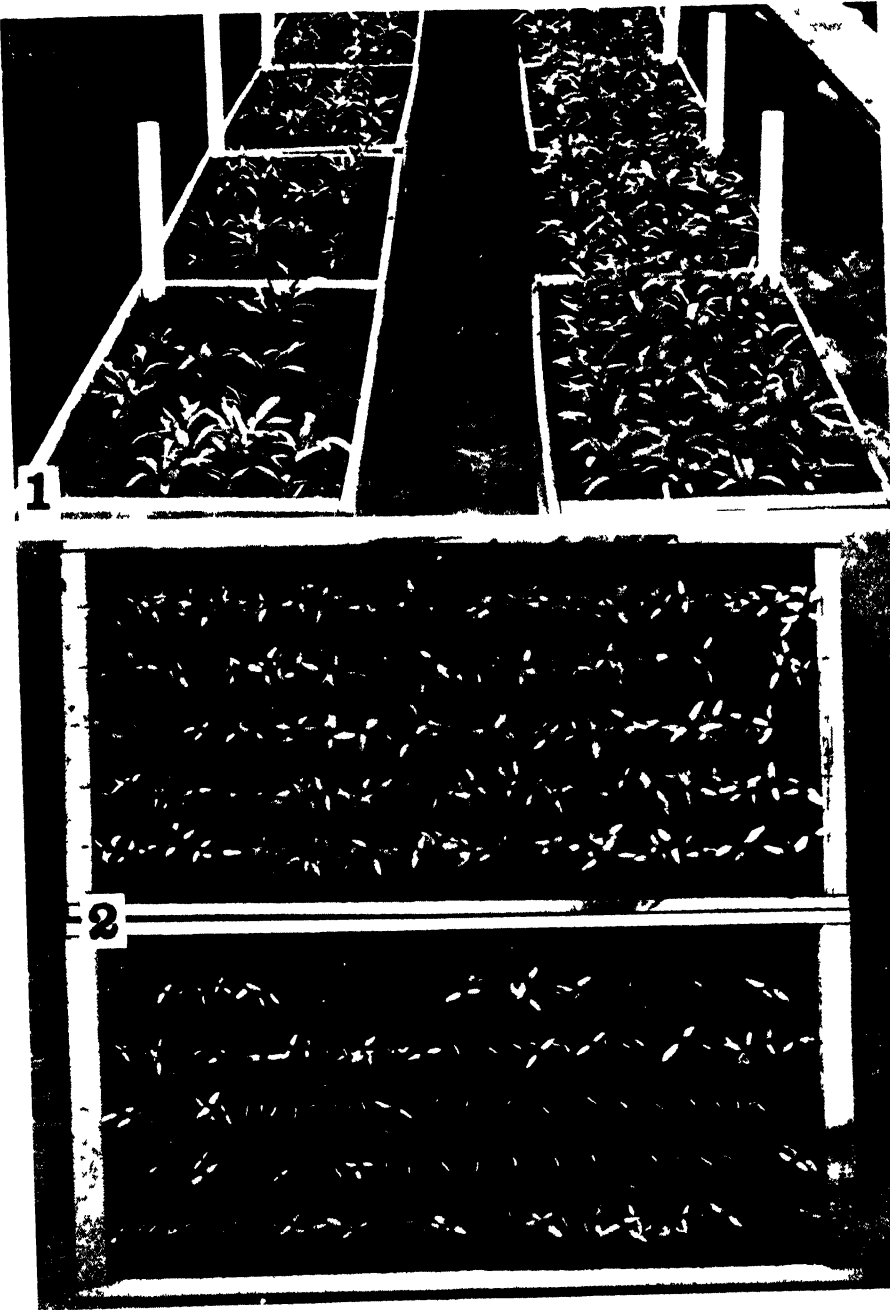
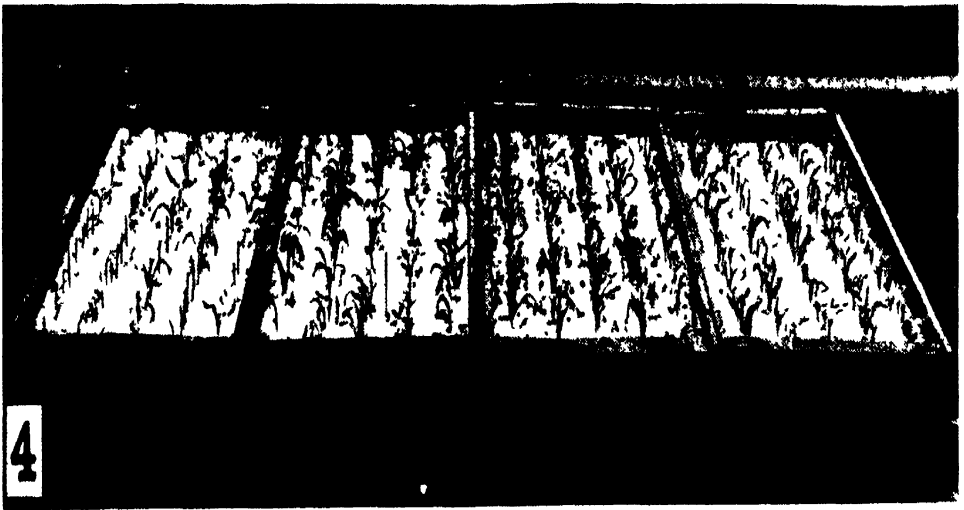


FIG. 1 Incidence of blackroot in relation to soil treatment with Arasan. All flats received 2 16-10 commercial fertilizer at 400 lb per acre. Flats to right received in addition, Arasan at 3 lb per acre applied by the precision method. FIG. 2 Effect of placement of Arasan on incidence of blackroot. In the top flat, the Arasan fertilizer mixture was mixed with the top  $1\frac{1}{2}$  in. of soil, in the lower one, the mixture was placed in the soil at a depth of  $1\frac{1}{2}$  in., the seed in both cases being planted  $\frac{1}{2}$  to  $\frac{3}{4}$  in. deep.



FIGS 3 and 4 Long-lasting effectiveness of Arasan treatment of soil in controlling blackroot FIG. 3 Surface-method treatment of flats, left to right, respectively, check, commercial fertilizer, fertilizer plus Arasan 3 lb per acre, and fertilizer plus Arasan 5 lb per acre These representative flats photographed Feb. 14, 1948 16 days after treatment of soil and first planting of seed on Jan. 29 FIG. 4 Same flats as above, replanted without further treatment, Mar. 4, and rephotographed Mar. 20, 1948 Thus, after 51 days Arasan treatment is still highly effective (Surface of soil covered with thin layer of white quartz sand for better photographic effects.)

other formulations containing tetramethylthiuram disulphide, or other compounds that might compare favorably with Arasan. With these considerations in mind, the various series of tests described below were planned and carried out.

### *I. Compatability of Arasan with Commercial Fertilizer*

Early in January, 1947, Canadian Industries Limited, Chatham, Ont., in anticipation of a future demand for a fertilizer with fungicidal properties, added Arasan to a 2-12-6 commercial fertilizer at the rate of 1 lb. of the former to 99 of the latter, the mixture then being placed in the company's storage bins. As prearranged, after intervals of 1, 2, 4, and 14 months, respectively, samples of the stored mixture were sent to the Harrow laboratory, where their effectiveness in controlling blackroot was compared with that of similar but freshly prepared mixtures of the fungicide and fertilizer. In order to obtain a 3 lb. per acre concentration of Arasan, it was necessary to apply the mixture at 300 lb. per acre. This meant a much heavier application of the fertilizer component than was employed in other tests. The experiments, carried out, as they were, with various periods of time intervening, had necessarily to be conducted under conditions that varied as to temperature and light intensity. The mixture was in all cases applied by the precision method and, as usual, four flats comprised a test unit for each treatment and check. In none of the tests of this series was the seed treated. In the last series of tests, *i.e.*, the series involving the 14-month-old mixture, 1947 seed instead of 1945 seed had to be used, since the supply of the latter had become exhausted. The soil used was the Brookston clay loam. Results obtained as well as other details in connection with these several tests are assembled in Table III.

The points of outstanding interest in the results shown in Table III are that (a) after being mixed with the fertilizer for as long as 14 months, Arasan had lost little of its fungicidal properties, and (b) the mixture of Arasan and fertilizer of various ages up to 14 months, though perhaps perceptibly less effective in controlling the disease than the freshly prepared mixture, was not significantly so. Such evidence would seem to be sufficient to establish the fact of compatability between the fungicide and fertilizer over a relatively long period of time. Commercial firms would thus be afforded ample opportunity of preparing mixtures of the two well in advance of when they might be required.

Table III shows that, in soil treated with the fertilizer alone, the incidence of pre-emergence damping-off, ranging as it does from 25.9% in the Feb.-March, 1947 tests to 48.2% in those of Apr.-May, 1948, was higher than might be expected. In this connection it should be pointed out that these and other tests were conducted in a greenhouse in which it was impossible to regulate the temperature other than by opening vents or closing valves. Thus, from mid-March, through April and early May, the sugar-beet flats were often subjected to overhead heat from the sun as well as that arising

TABLE III

EFFECT OF SOIL TREATMENT WITH 1- 2-, 4-, AND 14-MONTH-OLD ARASAN-FERTILIZER MIXTURES  
AS COMPARED WITH THAT OF SIMILAR BUT FRESHLY PREPARED MIXTURES  
ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Duration of experiment	Per acre soil treatment	Emergence, %*	Incidence of blackroot, %*	
			Pre-emergence phase (approx.)	Post-emergence phase
Feb. 3 to Mar. 10, 1947**	300 lb. 1-month-old (2-12-6 + Arasan) mixture	87.1	10.9	6.8
	300 lb. freshly prepared (2-12-6 + Arasan) mixture	89.4	8.6	5.3
	300 lb. 2-12-6	62.1	25.9	33.9
Mar. 3 to Apr. 7, 1947**	300 lb. 2-month-old (2-12-6 + Arasan) mixture	79.5	18.5	6.6
	300 lb. freshly prepared (2-12-6 + Arasan) mixture	87.4	10.6	4.8
	300 lb. 2-12-6	57.9	40.1	30.1
May 6 to June 10, 1947**	300 lb. 4-month-old (2-12-6 + Arasan) mixture	92.3	5.7	7.8
	300 lb. freshly prepared (2-12-6 + Arasan) mixture	88.6	9.4	6.1
	300 lb. 2-12-6	43.4	44.6	37.5
Apr. 6 to May 11, 1948***	300 lb. 14-month-old (2-12-6 + Arasan) mixture	103.3	5.7	7.4
	300 lb. freshly prepared (2-12-6 + Arasan) mixture	107.7	1.2	5.6
	300 lb. 2-12-6	60.8	48.2	35.0

\* Average of four flats.

\*\* 1945 and \*\*\*1946 seed, average germinating capacity 98 and 109%, respectively.

from the heating pipes. Under such conditions, the temperature not infrequently exceeded 100° F. for varying periods of time. This necessitated more frequent watering, with the result, as might be expected, that incidence of pre-emergence damping-off was higher than that ordinarily encountered in the field.

Table III shows also that, in soils treated with the Arasan-fertilizer mixture, incidence of postemergence blackroot ranged from 4.8 to 7.8%. If, as in previous tests, the seed had been treated, the incidence both of post- and pre-emergence damping-off would probably have been appreciably reduced.

## II. Simplification of the Method of Applying Arasan to the Soil

Coincidentally with the compatibility tests described above, attention was being directed towards finding a method of applying the Arasan-fertilizer mixture that could replace the highly impractical precision method. With this in view, a preliminary experiment was carried out in which fertilizer alone, and fertilizer with Arasan added at three different rates, namely, 3, 5, and

7 lb. per acre, respectively, were applied to the soil (Brookston clay loam) by the surface application method. In this experiment, four flats comprised the test unit for each of the four treatments and untreated check. Five rows of untreated 1945 seed, 44 per row, were planted in each flat. Thus, in the 20 flats comprising the test, a total of 4400 seeds were planted. The experiment, begun on Jan. 29, 1948, terminated five weeks later on Mar. 4. The results, as will be noted below, indicated that without any loss of effectiveness the surface application method might readily replace the precision method. However, to check this point more carefully, two additional series of tests, *B* and *C*, were carried out, in which the two methods were compared simultaneously. In these supplementary tests, 1947 instead of 1945 seed had to be used but otherwise the trials were carried out in as exact replication as possible. Details in connection with the three trials, including results obtained, are assembled in Table IV.

TABLE IV

COMPARATIVE EFFECTIVENESS OF PRECISION AND SURFACE-APPLICATION METHODS OF APPLYING ARASAN-FERTILIZER MIXTURE TO SOIL FOR CONTROL OF BLACKROOT

Duration of experiment	Per acre soil treatment		Method of application	Emergence, %*	Incidence of blackroot, %*		Av. no. of surviving seedlings per ft. of row*
	Arasan	Fertilizer (2-16-8)			Pre-emergence phase	Post-emergence phase	
<i>A</i> ** Jan. 29 to Mar. 4, 1948	7 lb.	200/200 lb.	Surface	79.0	18.0	2.0	18.6†
	5 lb.	"	"	87.2	10.8	4.7	19.9
	3 lb.	"	"	92.3	5.7	5.4	16.8
	Nil	"	"	46.3	51.7	47.6	5.7
	Nil	Nil		36.7	61.3	47.4	4.6
<i>B</i> *** Apr. 6 to May 11, 1948	3 lb.	200/200 lb.	Precision	102.6	6.4	16.8	20.3†
	3 lb.	"	Surface	103.3	5.7	11.2	22.0†
	Nil	"		101.9	7.1	35.0	10.1
<i>C</i> *** June 15 to July 20, 1948	4 lb.	Nil	Surface	109.0	Nil	7.2	24.2
	4 lb.	200, 200 lb.	Surface	113.5	Nil	2.7	26.3
	4 lb.	"	Precision	107.6	1.4	2.6	25.0
	Nil	"	Surface	70.4	43.5	31.6	11.7
	Nil	Nil		92.6	16.4	55.8	9.8

\* Average of four flats.

\*\* 1945 and \*\*\*1947 seed used, average germinating capacity 98 and 109%, respectively.

† Toxicity symptoms apparent.

As shown in Table IV and as illustrated in Fig. 3, the results of Expt. *A* indicated that the surface method of application of the Arasan-fertilizer mixture might be quite as effective as the precision method in controlling blackroot. When Arasan was applied at 5 or 3 lb. per acre by the new method, loss of seedlings due to pre-emergence damping-off was relatively low, while that due to the postemergence phase of the disease did not exceed 5.4%.

These losses are in marked contrast to those recorded for soil that had not been treated or to which only fertilizer had been added. A 7 lb. per acre application of Arasan reduced emergence and induced marked toxicity symptoms on many seedlings.

In Expt. *B*, in which for the first time the two methods were tested simultaneously, the incidence of blackroot was lowest in both phases of the disease, following the surface method of application. It will be noted that, for both methods, the incidence of postemergence blackroot was unusually high. The reason for this was explained in the preceding section, namely, excessively high temperature necessitating more frequent watering during a critical period of the experiment. In this connection, it should be pointed out that on 13 days during the earlier part of the experiment, when the greenhouse had still to be heated artificially, the temperature, for periods varying from one to several hours, ranged from 90° to 100° F., and, on nine days, exceeded 100°, approaching a maximum of 110° on the three successive days (9th, 10th, and 11th days after planting) during which the seedlings were in the most susceptible stage. In this experiment, there were indications also of Arasan injury, as evidenced not only by typical foliar symptoms but also by some burning of the roots. The possibility that, at high soil temperatures, Arasan may prove to be more or less phytotoxic was confirmed by experiments that are described later in the present paper.

In Expt. *C*, as the data indicate, the fact of the surface application method being as efficient as the precision method was confirmed.

In Table IV, as will be noted, a new criterion for evaluating the effectiveness of the addition of Arasan to the soil has been added, namely, a comparison of the number of surviving seedlings per foot of row in treated and nontreated soil. At the very least, the ratio is two to one (Expt. *B*) in favor of the treated soil and is still higher in the other two experiments.

### *III. Correct Placement of Arasan*

Of the many points to be considered in the treatment of soil with Arasan, none is more important, especially from the standpoint of field practice, than that of proper placement of the protectant. As has already been pointed out in the introductory paragraphs, under field conditions, the postemergence phase of the disease is, in general, more to be feared than the pre-emergence phase. In the postemergence phase, the hypocotyl of the seedling is attacked at or near the soil level. Theoretically, it would seem best to so place the Arasan that it would not only surround the seed but would also be incorporated in the soil between the seed level and the surface of the soil. Such placement should afford protection against pre- and postemergence phases of the disease. To test the validity of this postulation, an experiment was carried out in which, in certain flats, by employing the surface method of application, the Arasan-fertilizer mixture was thoroughly mixed in the top 1½ in. of soil (Brookston clay loam). The seed was then planted to a depth

of  $\frac{1}{2}$  to  $\frac{3}{4}$  in. In a corresponding number of flats, an Arasan-fertilizer mixture of the same concentration was applied to the soil at a depth of  $1\frac{1}{4}$  in. below the surface, the seed again being planted  $\frac{1}{2}$  to  $\frac{3}{4}$  in. deep. The test was repeated and the results of the two trials are recorded in Table V.

TABLE V  
EFFECT OF PLACEMENT OF ARASAN IN SOIL ON EMERGENCE OF  
SEEDLINGS AND INCIDENCE OF BLACKROOT

Duration of experiment	Per acre soil treatment		Placement of Arasan	Emergence, %*	Incidence of blackroot, %*	
	Arasan	Fertilizer (2-16-8)			Pre-emergence phase (approx.)	Post-emergence phase
June 15 to July 20, 1948	4 lb.	200/200 lb.	Top inch of soil	113.5	Nil	2.7
	4 lb.	"	Depth of $1\frac{1}{4}$ in.	62.9	46.1	40.4
	Nil	"		82.3	26.7	17.0
	Nil	Nil		92.6	16.4	55.8
Aug. 17 to Sept. 21, 1948	4 lb.	200/200 lb.	Top inch of soil	114.0	Nil	1.1
	4 lb.	"	Depth of $1\frac{1}{4}$ in.	77.7	31.3	21.6
	Nil	Nil		58.6	50.4	46.3

\* Average of three flats.

As Table V shows, when the seed germinated in, and the seedling grew up through soil with which had been incorporated the Arasan-fertilizer mixture, the incidence of disease was virtually negligible. In marked contrast, as is shown in Fig. 2, when the mixture was placed below the seed and the seedling grew up through the soil without the protection of Arasan, the incidence of both phases of damping-off was high. These tests, while admittedly on a limited scale, demonstrate conclusively, nevertheless, not only the importance of adding Arasan to infested soil but also the necessity of so placing it that best results may be obtained. The implications as relating to field practice are of especial importance.

#### IV. Effectiveness of Arasan in Soils of Different Types

As mentioned in an earlier section, blackroot has been observed to cause striking loss of seedling stands in at least three quite different types of soil, namely, Brookston clay loam, 'Exeter' sandy loam, and dark-colored clay of the Clyde series. It was a matter both of interest and of importance to find out whether and to what extent Arasan might control the disease in these different soils, the more especially since it was known that different pathogens were associated with each. Consequently, using 1945 seed and employing the precision method of application, tests were set up with results as shown in Table VI.



TABLE VI

EFFECTIVENESS OF ARASAN TREATMENT OF SOILS OF DIFFERENT TYPE ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Soil type	Predominating pathogen(s)	Treatment			Emergence, %*	Incidence of blackroot, %*	
		Seed	Soil			Pre-emergence phase (approx.)	Post-emergence phase
			Arasan	Fertilizer			
Brookston clay loam	<i>Aphanomyces cochlioides</i>	½ % Arasan	4 lb.**	200/200 lb.**	96.0	2.0	1.0
		Nil	4 lb.	"	91.8	6.2	2.0
		Nil	Nil	"	76.1	21.9	65.0
'Exeter' sandy loam	<i>Pythium aphanidermatum</i>	½ % Arasan	4 lb.	200/200 lb.	103.4	Nil	Nil
		Nil	Nil	"	63.6	34.4	69.0
Clyde clay	<i>A. cochlioides</i> <i>Pythium ultimum</i>	½ % Arasan	4 lb.	200/200 lb.	102.7	Nil	Nil
		Nil	Nil	"	78.4	19.6	6.5

\* Average of four flats.

\*\* Per acre applications.

As Table VI shows, the Arasan treatment was effective in reducing the incidence of the disease in all three types of soil, the more especially so, however, in the case of the Brookston clay and the 'Exeter' sandy loam.

#### V. Comparative Effectiveness of Arasan and Other Formulations Containing Tetramethylthiuram Disulphide as the Lethal Agent

The only other formulations containing tetramethylthiuram disulphide as the active ingredient that were tested were Tersan and a product of Naugatuck Chemicals, Division of Dominion Rubber Company, Elmira, Ont. This product was labelled "Control D-419, tetramethylthiuram disulphide-50% non-wettable". As contrasted with the pink color of Arasan, D-419 is white and it also has a different odor. Thus, it would appear that the two products might differ as to their inert ingredients. In the tests, Tersan, like Arasan and D-419, was applied in the dry powder form. Details in connection with these tests together with results obtained are assembled in Table VII.

As Table VII shows, the results obtained with Arasan and D-419 were, in the aggregate, appreciably better than those with Tersan, the apparent superiority of the two first-mentioned compounds being more noticeable in the control of the postemergence phase of the disease. According to the results as tabled, there would seem to be little choice between Arasan and D-419. However, certain observations made during the tests reveal some interesting possibilities with respect to D-419. In the Jan. 13-Feb. 17 test, for example, emergence of seedlings for treatments and checks began on the

TABLE VII

COMPARATIVE EFFECTIVENESS OF TREATMENT OF SEED AND SOIL, OR OF SOIL, WITH ARASAN, TERSAN, AND D-149, ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Duration of experiment	Treatment			Emergence, %*	Incidence of blackroot, %*	
	Seed	Soil			Pre-emergence phase (approx.)	Post-emergence phase
		Protectant	Fertilizer (2-16-10)			
Mar. 3 to Apr. 7, 1947***	Nil	Tersan, 3 lb.**	200/200 lb.**	88.5	9.5	5.7
	"	Arasan, 3 lb.	"	90.5	7.5	0.6
	"	Nil	"	49.7	46.3	51.1
Jan. 13 to Feb. 17, 1948***	Nil	Tersan, 3 lb.	200/200 lb.	82.3	15.7	6.7
	½ % Arasan	Arasan, 3 lb.	"	85.9	12.1	2.7
	½ % D-419	D-419, 3 lb.	"	78.4	19.6	4.8
	½ % D-419	D-419, 4 lb.	"	82.5††	15.5	2.7
	Nil	Nil	"	62.3	35.7	41.4
June 15 to July 20, 1948†	Nil	Arasan, 4 lb.	200/200 lb.	110.5	Nil	2.7
	"	D-419, 4 lb.	"	116.5††	Nil	0.5
	"	Nil	"	82.3	26.7	17.0

\* Average of three flats.

\*\* Per acre applications.

\*\*\* 1945 and † 1947 seed used, average germinating capacity 98 and 109%, respectively.

†† Toxicity symptoms apparent.

seventh day after planting the seed. However, first- and second-day emergence for the D-419 treatments was much higher than for those for Arasan or check, as the following figures disclose:

Treatment	Emergence, days after planting, %	
	Seventh day	Eighth day
Arasan, 3 lb.	1.5	18.4
Check	1.3	17.1
D-419, 3 lb.	9.5	50.7
D-419, 4 lb.	7.5	46.2

It was also observed subsequently that the seedlings in the soil receiving the 3 lb. per acre application of D-419, were outstanding in vigor of growth. In the flats receiving the 4 lb. per acre application of D-419, many of the seedlings exhibited toxicity symptoms that corresponded in severity with those on seedlings grown in soils that had received slightly heavier applications of Arasan. From these observations it would appear that D-419 is slightly 'stronger' than Arasan. Since, however, each contains 50% of the active ingredient, any difference in 'strength' would seem to be inherent in the 'inert' ingredients. In any case, the efficacy of D-419 was such as to warrant rating it at the very least on a par with Arasan.

# VI. Period of Time During Which Arasan Remains Effective in the Soil

Often, under field conditions, a period ensues just after planting, when, as the result of unfavorable environmental conditions, germination or emergence is delayed and the seeds or seedlings need added protection against the soil-borne pathogens. Under such adverse conditions, the prime requisite of a protectant is that its protective action be extended over a long enough period of time during the critical period to ensure an adequate stand of seedlings. In order to gain some information as to how long Arasan might remain effective in the soil, a few exploratory experiments were carried out. In an earlier experiment in which 1945 seed was used, the Arasan was applied by the precision method, three weeks prior to planting, to lots of Brookston clay soil that meanwhile were kept wet and dry, respectively. In a later experiment, at the conclusion of a routine five-week test in which the Arasan had been applied by the surface method of application, the seedlings were removed and, with no additional treatment of the soil, a replicate planting was made by pushing the seeds to a depth of  $\frac{1}{8}$  in. in the soil, along the original rows. Details in connection with these experiments together with results obtained are recorded in Table VIII.

TABLE VIII

EFFECT OF PREPLANTING TREATMENT OF SOIL WITH ARASAN, ON EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Expt.	Soil treatment					Emergence, %*	Incidence of blackroot, %*	
	Arasan application			Moisture condition of soil prior to application	Fertilizer (2-16-10)		Pre-emergence phase (approx.)	Post-emergence phase
	Rate	Method	Time					
I***	3 lb.**	Precision	Three weeks before planting	Dry, 3 weeks	200/200 lb.**	91.7	6.3	
	3 lb.	"	"	Mod. wet, 3 weeks	"	86.0	14.0	
	3 lb.	"	At planting	Dry, 3 weeks	"	100.6	Nil	
	3 lb.	"	"	Mod. wet, 3 weeks	"	100.6	Nil	
	Nil				"	60.2	27.8	
II†	5 lb.	Surface	Five weeks before planting	Mod. wet, 5 weeks	200/200 lb	116.3	Nil	2.9
	3 lb.	"	"	"	"	115.2	Nil	6.8
	Nil				"	71.0	38.0	23.6
	Nil				Nil	62.4	36.6	38.2

\* Average of four flats.

\*\* Per acre applications.

\*\*\* 1945 and † 1947 seed, average germination 98 and 109%, respectively.

In Expt. I, as Table VIII shows, Arasan applied at planting time afforded more complete protection against the disease than when applied three weeks prior to planting. In the wet soil the incidence of disease was appreciably

higher than in the corresponding dry soil. The results of Expt. II are especially interesting. After effectively protecting an earlier stand of seedlings (see Table IV, Expt. A and Fig. 3), the Arasan at both 5 and 3 lb. per acre, afforded, virtually, equally as high protection to those of the replicate planting (see Fig. 4). The latter was made on Mar. 4, 1948. First emergence was noted on Mar. 9 and the period of highest seedling mortality was from Mar. 11 to 14, inclusive. The Arasan had been originally applied on Jan. 29. Thus, after a period of at least 45 days, the effect of Arasan had not been dissipated. These experiments, while admittedly limited both as to number and scope, indicate clearly, nevertheless, that Arasan may be expected to be effective over relatively long periods of time.

### *VII. Effect of Temperature and Moisture on Arasan-treated Soil*

All the experiments described above had been carried out under conditions that in a number of instances permitted relatively wide variations as to both temperature and moisture of the soil. In certain of the trials, it had been indicated that each of these variables—more particularly temperature, perhaps—had not been without effect in modifying either the fungicidal or the phytotoxic possibilities of Arasan. Under these circumstances, it seemed advisable to test the protectant under conditions of more precise control of the two variables in question. For such tests there were available three Wisconsin temperature tanks with cannisters 18½ in. deep and 7½ in. in diameter. A cannister was prepared as follows. First, two flower pots, each of 6½ in. depth and 6½ in. diameter, one inverted over the other, were set in a cannister. Then, sufficient gravel to fill in around the pots and to cover them to a depth of ½ in. was added. Meanwhile, a quantity of the naturally-infested Brookston clay soil had been sieved through a screen of ¾ in. mesh. A 2 in. layer of this soil was placed on top of the gravel. An amount of fertilizer representing one-half of the quantity to be used was then applied and an inch of Arasan-treated rootrot soil was added. The second half of the fertilizer was then applied and, lastly, another inch of the Arasan-treated soil added, the top of the latter being 1¾ in. below the rim of the cannister. Seed (1945) to the number of 60 per cannister was pushed to a depth of ¾ in. in the top layer of the treated soil. Eight cannisters were prepared for each tank in this way, except that in the four that were to serve as checks, the rootrot soil was not treated with Arasan. The amounts of both Arasan and fertilizer requisite for the cannister type of container were carefully calculated from the data available from the many previous experiments. One of the three tanks was operated at 14° to 16° C., another at 19° to 21° C., and the third at 27° to 29° C. An attempt was made to maintain the soil in all cannisters at a uniformly moderate level of moisture. To do this, it was found necessary to add 150 cc. of water three times a day to each cannister of the high temperature series, 50 cc. once daily to each of those of the intermediate temperature, and smaller amounts at longer intervals to the low temperature one. Two tests, one beginning Nov. 20, 1947, the other Jan. 12, 1948, were completed. The same

technique was used in both but, in the second, the seed was treated as well as the soil, the amount of Arasan added to the soil being at the rate of 3 lb. per acre. Details in connection with these tests together with the results obtained are assembled in Table IX.

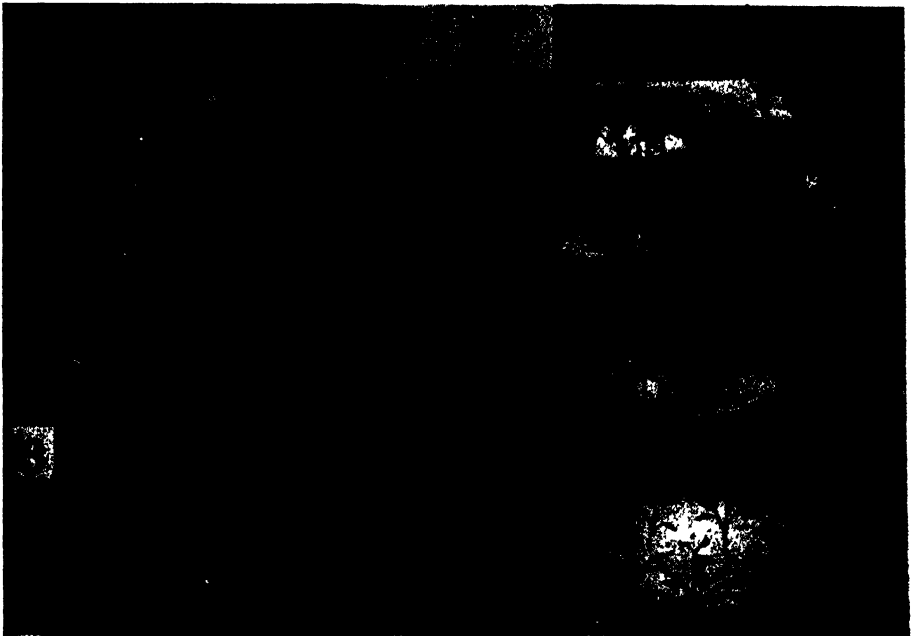
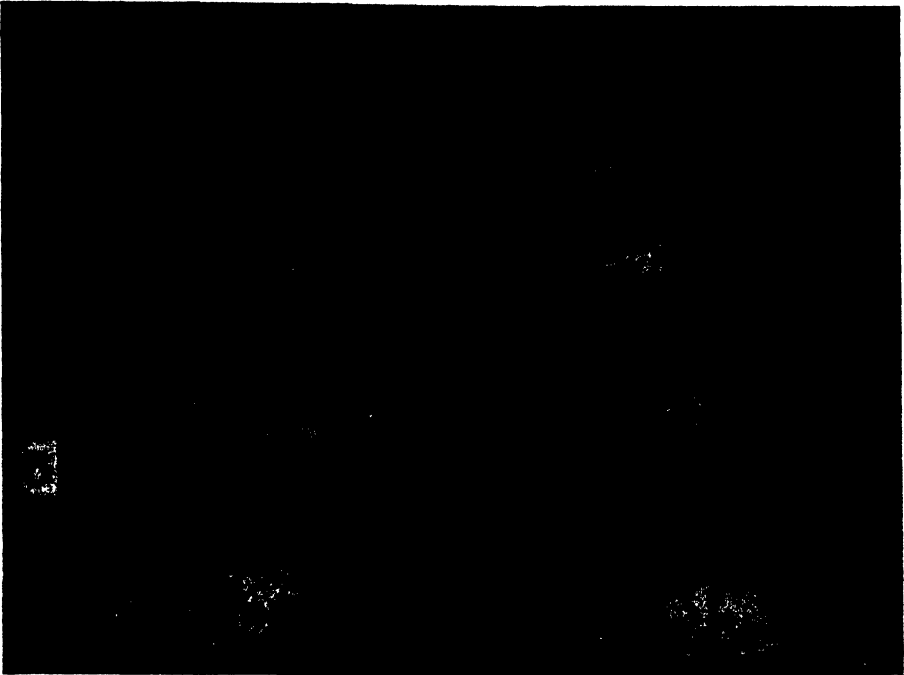
TABLE IX

EFFECT OF TEMPERATURE AND MOISTURE ON ARASAN-TREATED SEED AND SOIL, AS INDICATED BY EMERGENCE OF SEEDLINGS AND INCIDENCE OF BLACKROOT

Soil temperature	Expt.	Treatment			Emergence, %*	Arasan toxicity	Incidence of blackroot, %*	
		Seed	Soil				Pre-emergence phase (approx.)	Post-emergence phase
			Arasan	Fertilizer (2-16-10)				
27° to 29° C.	1	Nil	4 lb.	200/200 lb.	87.9	Severe Moderate	10.1	
	2	$\frac{1}{2}$ % Arasan	3 lb.	"	85.7		12.2	2.9
	1	Nil	Nil	"	57.9		40.1	32.4
	2	Nil	Nil	"	61.2		36.8	25.8
19° to 21° C.	1	Nil	4 lb.	"	87.9		10.1	4.2
	2	$\frac{1}{2}$ % Arasan	3 lb.	"	75.0		23.0	3.3
	1	Nil	Nil	"	43.7		54.3	31.4
	2	Nil	Nil	"	62.9		35.1	19.2
14° to 16° C.	1	Nil	4 lb.	"	91.2		7.8	4.1
	2	$\frac{1}{2}$ % Arasan	3 lb.	"	84.5		13.5	0.4
	1	Nil	Nil	"	47.0		51.0	24.7
	2	Nil	Nil	"	47.9		40.1	13.0

\* Average of four cannisters.

So far as the main interests of this paper are concerned, the most important point, as revealed by Table IX and as clearly indicated in Figs. 5 and 6, is that, up to a temperature of 21° C. (= 70° F.), at least, Arasan applied at a rate as high as 4 lb. per acre will, in a moderately wet soil, effectually control blackroot, without injury to the seedlings, the extent of control of the post-emergence phase of the disease under these circumstances being especially noteworthy. At 27° to 29° C., moderate injury resulted from a 3 lb. per acre application of Arasan to the soil. This injury, scarcely discernible on the foliage, appeared as a burning of the roots and lower hypocotyl, affected tissues also exhibiting a tendency towards flaccidity. As a result of the 4 lb. per acre application of the protectant, the types of injury just described were accentuated. In addition, typical foliar symptoms were noted, the injury in a number of seedlings being so severe as to cause their death. That the types of injury observed could in no way be ascribed to the fertilizer was proved by the fact that the roots of the surviving seedlings in the similarly fertilized check soil were turgid and healthy. That Arasan will injure the roots of seedlings if the temperature is high enough is a point of academic



FIGS. 5 and 6. Effectiveness of Arasan treatment under conditions of controlled temperature and moisture of the soil. All cannisters received 2-16-10 commercial fertilizer at 400 lb. per acre. Cannisters to left received in addition Arasan at 4 lb. per acre applied by the precision method. (Surface of soil covered with thin layer of white quartz sand for better photographic effects.)



interest rather than one of practical significance, because rarely, if ever, would the temperature of the soil at the time that sugar beets are normally planted reach or remain long enough at the danger point for injury to take place.

*Other Proprietary Materials in Comparison with Compounds Containing Tetramethylthiuram Disulphide*

In the present studies, some 11 other proprietary materials in addition to Arasan, Tersan, and D-419, were tested as to their effectiveness in controlling blackroot. While some of these were found to be totally inadequate for the purpose, certain others, including Mycotox No. 1 (2, 4, 5-trichlorophenyl acetate), C-119 (copper trichlorophenate, 20%), CCH-358 or 358A, and F-800 (2,4,5-trichlorophenyl chloroacetate, 50%)—the three latter supplied by the Dow Chemical Company—showed encouraging possibilities. C-119 gave especially good results in greenhouse experiments but, when tested later under field conditions, failed to afford adequate protection to the seedlings. Under cooler greenhouse conditions, CCH-358 or 358A, and F-800 compared favorably with Arasan, but, when the tests were repeated under higher temperature conditions, their effectiveness was greatly diminished. In regard to these products, there is some evidence to suggest that they are fungistatic rather than fungicidal in their capabilities.

### Discussion

Since blackroot of sugar beets is so essentially a field problem, methods and materials used in greenhouse investigations, such as those recorded above, must be evaluated primarily from the standpoint of the possibility of their adaptability to field practice under field conditions. Moreover, in consideration of the extremely wide difference in precision that must necessarily exist between field and greenhouse methods, the results obtained in greenhouse experiments must be of such a convincing nature as to indicate the probability of a reasonable measure of success from analogous field experiments. If the materials and methods employed in the experiments described in this paper, as well as the results obtained, are examined in the light of the above considerations, there would seem to be suggested a particularly promising avenue of approach to the remedying or control of the disease in question. In the first place, the question of the materials involved offers no serious problem to those concerned. Proprietary materials, such as Arasan or D-419, containing the necessary active agent, tetramethylthiuram disulphide, are readily available to the trade and can be mixed with commercial fertilizers well in advance of the time the mixture may be required for use. Since no appreciable benefit is to be derived from seed treatment, that process can be eliminated. The grower receives the fungicide-fertilizer mixture all ready for use. Many growers already possess—or can be furnished with—the type of sugar-beet machinery with which the mixture can be successfully applied; provided that



care is exercised as to correct rate of application and proper placement of the protectant.\* The row application method and the relatively small amount of the protectant required would make it economically possible for almost any grower to reap advantage of the benefits to be derived from the treatment. Even if the sugar-beet seed is delayed in germination, the grower may still expect the seedlings to be protected, since Arasan, for example, has been found to remain effective in the soil over relatively long periods of time. In fact, this is probably one of the more important reasons why the materials containing tetramethylthiuram disulphide proved to be so much more effective than many of the other materials tested. Admittedly, under certain combinations of circumstances, the treatment may be expected to lose some of its effectiveness. If, for example, when seedlings are in the stage at which they are most susceptible to the disease, the temperature and moisture of the soil should both be excessively high—more particularly the temperature, perhaps—then, the treatment may not afford sufficient protection to ensure an adequate and uniform stand of seedlings. Such a combination of unfavorable circumstances would be more likely to be encountered in late plantings of sugar beets.

When the data tabulated above are examined and compared, it will be found that, invariably, regardless of experimental procedure, emergence of seedlings was greater and incidence of blackroot was lower in Arasan-treated than in nontreated rootrot soil, the difference resulting from treatment with the chemical being so great as to indicate significance without recourse to statistical analysis. The differences were of such a consistent and decisive nature as to suggest also the high probability of success of the methods and materials involved when applied in field practice.

As a recent report by Leach and Snyder (9) suggests, workers elsewhere are becoming interested in the possibilities of localized chemical applications to the soil for the control of soil-borne diseases of crops other than sugar beets.

### Acknowledgments

For supplying seed, for transporting soil, and for ready co-operation in many other ways, the writers express appreciation to the Canada and Dominion Sugar Company, Chatham, Ont. For supplying chemicals, especial thanks are due Canadian Industries Limited, Chatham, Ont.; Dow Chemical Company, Midland, Mich.; and Naugatuck Chemical Division, United States Rubber Company, Elmira, Ont.

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\* This statement is based on the results of field experiments that have already been carried out but an account of which will be reserved for a complementary paper.

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## INTERVARIETAL CROSSES IN *PUCCINIA GRAMINIS*<sup>1</sup>

By T. JOHNSON<sup>2</sup>

### Abstract

Crosses made in all possible combinations between varieties *Tritici*, *Secalis*, *Avenae*, *Agrostidis*, and *Poa* of *Puccinia graminis* Pers. have shown the existence of considerable intersterility in crosses between some of the varieties. Fertility was relatively high in crosses between vars. *Tritici* and *Secalis*, *Avenae* and *Agrostidis*, *Avenae* and *Poa*, and *Agrostidis* and *Poa*. Fertility was much lower in crosses between vars. *Tritici* and *Avenae*, *Secalis* and *Avenae*, and *Secalis* and *Poa*. In crosses between certain pairs of varieties, the degree of fertility varied according to the direction of the cross. *Tritici* × *Agrostidis*, *Tritici* × *Avenae*, *Tritici* × *Poa*, *Secalis* × *Agrostidis*, and *Secalis* × *Poa* crosses succeeded much better than the reciprocal crosses. Sporidia from the  $F_1$  teliospores of *Tritici* × *Secalis* hybrids infected barberry with the production of normal pycnia and aecia. Sporidia of *Tritici* × *Avenae* and *Secalis* × *Avenae* hybrids produced, on barberry, infections with few pycnia and these excreted little nectar; no aecia were formed but urediospores and teliospores were occasionally produced in old infections. Generally, an  $F_1$  hybrid rust possessed a wider host range than did either of the parent varieties; but the pathogenicity of the hybrid on a given host was less than that of the parent variety that attacked that host. In hybrids of varieties that differed markedly in urediospore size, the urediospores of the  $F_1$  hybrid rusts were intermediate in size between those of the parents. The pathogenic characteristics of the  $F_2$  (uredial) generation were studied only in *Tritici* × *Secalis* hybrids. Variation in pathogenicity was rather restricted. The races isolated were moderately pathogenic to barley and weakly pathogenic to wheat and rye.

### Introduction

Stem rust (*Puccinia graminis* Pers.) is specialized into several varieties that differ little morphologically but show striking pathogenic differences. In North America, the following five varieties are known to occur on graminaceous hosts and on barberry: *Tritici*, *Avenae*, *Secalis*, *Agrostidis*, and *Poa*. A sixth variety, *Phlei-pratensis*, also occurs on grass hosts but has not been shown to infect barberry in America though its ability to infect that host in Europe has been reported (11).

Work already published (4, 5, 8, 10) has shown that crosses may be made between the varieties *Tritici* and *Avenae*, *Tritici* and *Secalis*, and *Tritici* and *Agrostidis*. However, the work thus far reported is not extensive enough to establish the degree of interfertility of the above-mentioned varieties or to indicate conclusively what pathogenic and other properties are to be expected

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in the progeny of the crosses. The work described in the present paper was undertaken to provide further information on these matters and particularly to study crosses between varieties that hitherto had not been crossed.

### Terminology

The technical terms used in connection with cereal rust studies are more or less familiar to all phytopathologists. There is, nevertheless, a possibility that such terms may be used in a somewhat different sense by different investigators. To avoid misunderstanding, definitions are given of the exact sense in which a number of these terms are used in this paper.

The word "variety" is used to signify the *forma specialis* of Eriksson.

The word "pustule" is used with reference to all spore forms of the rust except the sporidia (basidiospores). In connection with the spore forms on barberry, it refers to infections bearing a single group of pycnia (pycnial pustules) or aecia (aecial pustules). In the spore forms on grass hosts, it refers to a single sorus of urediospores or teliospores.

"Cross" refers to the transfer of the exudate or nectar from the pycnia (which contains the pycniospores) of one or more pycnial pustules of one variety of the rust to a single haploid pustule of another variety. In the sense used, a cross does not necessarily imply the production of aeciospores. A dozen crosses may be made and possibly only one or two may succeed in producing aecia. In crossing any two varieties of the rust, the crosses are made in opposite directions according to which variety is used as the "female", i.e., nectar-receiving parent. A cross reported as *Agrostidis* × *Avenae* signifies that a haploid *Agrostidis* pustule received an application of *Avenae* pycnial nectar. *Avenae* × *Agrostidis* indicates the reverse procedure. Combinations without reference to the direction in which crosses were made are occasionally referred to as *Tritici-Agrostidis*, *Avenae-Secalis*, etc.

The term "hybrid" is used to signify the dikaryon formed when, as a result of a cross, a haploid nucleus of one rust variety comes into association with a haploid nucleus of another variety. This practice is open to objection by those who maintain that the hybrid does not come into being until the fusion of these two nuclei in the teliospore. A distinction could be made by regarding the dikaryon as an "associant" or a "pseudohybrid" or a "prohybrid"; but in the present paper the attitude is taken that such a distinction is unnecessary as it is known that the rust dikaryon does display the essentials of hybridity, such as dominance, recessiveness, and intermediacy. In other words, the genes in the dikaryon act as if they were present in a diploid nucleus.

### Materials and Methods

In the varieties *Tritici* and *Avenae*, known physiologic races of ascertained purity were used in the crosses, the teliospores being produced on wheat or oat plants grown in the greenhouse. In the varieties *Secalis*, *Agrostidis*,

and *Poa*, the teliospores employed were collected in nature on grass hosts congenial to these three varieties. Data on the actual collections used are given below:

Variety	Host	Date of collection	Locality
<i>Secalis</i>	<i>Agropyron repens</i>	May, 1942	McKellar, Ont.
"	"	May 13, 1944	"
"	"	May 23, 1945	Fredericton, N.B.
<i>Agrostidis</i>	<i>Agrostis alba</i>	Sept. 10, 1943	Ottawa, Ont.
"	Same source, greenhouse-formed telia		
"	<i>Agrostis</i> sp.	May 23, 1945	Fredericton, N.B.
"	" <i>lenuis</i> , <i>A. Reuteri</i> , <i>A. alba</i>	April, 1946	"
<i>Poa</i>	<i>Poa compressa</i>	May 13, 1944	Ottawa, Ont.
"	Same source, greenhouse-formed telia		
"	<i>Berberis vulgaris</i>	1945	Guelph, Ont.
	Greenhouse-formed telia on <i>Poa compressa</i>		

In the collections of telia made in nature, precautions were taken to ensure, as far as possible, that the correct variety was used in each case. As each collection was made or received, telia-bearing straws with heads or panicles attached were separated out, and so long as they were available only such straws were used for the infection of barberry. Later, it became necessary, in certain of the collections, to use straws with no heads attached. But whether or not heads were attached, the aeciospores that developed were tested for their infectivity on wheat, oats, rye, barley, and the required grass hosts to secure evidence that the correct variety of the rust was being used.

As a sparse distribution of pycnial pustules is desirable for crossing purposes, attempts were made to inoculate barberry plants in such a way that the infections were widely distributed over the leaves and separated from each other by a distance of at least one centimeter. Infection of barberry was accomplished by suspending the telia-bearing straws over a plant enclosed in a lantern chimney, the wet straws being pressed firmly against a blotting paper in a Petri plate inverted over the top of the chimney. The length of time the telia were suspended over the plants varied from a few hours to 24 hr. or more, depending on the rate of sporidial discharge as determined prior to inoculation.

After inoculation, the infected plants were kept in close-mesh screen cages for at least three weeks, or until development of aecia was general. It was then assumed that isolated pustules that had not produced aecia were haploid, and these were therefore selected for use in the crossing studies. Each of the selected pustules on a plant was given a number (inscribed in India ink on the adjacent leaf area), and thereafter diagrams showing the location of each pustule were entered in the record book.

The application of pycnial nectar of one variety of the rust to pustules of another variety was then carried out by means of a platinum wire loop, which was sterilized between successive transfers by passing through a flame. In most crosses, nectar was transferred from many pustules of one variety of the rust to each individual pustule of another variety so as to favor as far as possible the formation of aecia in each nectar-receiving pustule. Following the transfer of nectar, the plants were replaced in the screen cages and the nectar-receiving pustules were examined every two or three days to record the development or nondevelopment of aecia.

When the aecia of any given pustule had reached appropriate development, they were removed by means of a small, sterile, metal spatula and placed in a drop of water on a sterile glass slide. There they were crushed to liberate the aeciospores and the resulting spore suspension was immediately applied to seedling leaves of the appropriate cereals—wheat and oats in the *Tritici-Avenae* crosses, wheat and rye in the *Tritici-Secalis* crosses, etc. Cultures thus established were propagated on the most suitable host and studied for their pathogenicity toward cereal varieties and grasses.

### Fertility Between Varieties of the Rust

It was shown by Craigie (1, 2, 3) that haploid pustules of *Puccinia graminis*, as also of *P. Helianthi* Schw., fell into two sex or interfertility groups, which he designated as (+) and (-). Application of pycniospore-bearing nectar of a (+) pustule to a (-) pustule, or vice versa, resulted in the production of aecia in the pustule receiving the application, providing both pustules belonged to the same variety of *P. graminis*.

In crosses between two physiologic races of the same variety of *P. graminis*, there appears to be little if any intersterility. When nectar is transferred from a haploid pustule of one physiologic race to several haploid pustules of another, the usual result is that about half of the nectar-receiving pustules produce aecia. If composite nectar derived from many pustules of one race, and therefore containing both (+) and (-) pycniospores, is applied to a number of pustules of another race, aecia are usually formed in all pustules receiving the application. In either case it may be said that fertility between the two races is complete. The results of numerous intravarietal crosses in the *Tritici* variety and in the *Avenae* variety indicate that within these varieties fertility between the different physiologic races is of a relatively high order. Crosses between different *Tritici* races, involving 164 haploid pustules, indicated 72% interfertility, and crosses between *Avenae* races, in which 137 pustules were employed, showed 100% interfertility.

In crosses between varieties of stem rust (intervarietal crosses), there is, on the other hand, evidence of a considerable degree of intersterility except possibly in *Tritici-Secalis* and *Agrostidis-Poae* crosses. Stakman, Levine, and Cotter (10) obtained no aecia as a result of the application of *Tritici* nectar to haploid *Agrostidis* pustules but obtained aecia in all *Tritici* pustules

that received *Agrostidis* nectar. Johnson, Newton, and Brown (4) applied nectar of haploid *Tritici* pustules to 36 haploid *Agrostidis* pustules with the result that only three produced aecia, which in all three cases were attributed to fortuitous selfing of the *Agrostidis* variety. Aecia of hybrid origin were, however, obtained in one of the 14 *Tritici* pustules receiving applications of *Agrostidis* nectar. With regard to *Tritici-Agrostidis* crosses, the published evidence indicates a considerable degree of intersterility at least on the *Agrostidis* side, i.e., when haploid *Agrostidis* pustules receive *Tritici* nectar. Johnson and Newton (5) also furnished some evidence of intersterility in *Tritici-Avenae* crosses. Aecia were formed in only one of 13 haploid *Avenae* pustules receiving a mass transfer of nectar derived from many *Tritici* pustules, and similarly aecia were formed in only one of 19 *Tritici* pustules that received *Avenae* nectar.

To the above-mentioned data may now be added similar information derived from the crosses under consideration in the present paper. In Table I a summary is given of all intervarietal crosses made by the writer, and, in addition, the *Tritici-Secalis*, *Tritici-Avenae*, and *Tritici-Agrostidis* crosses mentioned above (4, 5). In the great majority of the crosses, a mass transfer of nectar was made from many pustules of one variety of the rust to individual haploid pustules of another variety, i.e., (+) and (-)  $\rightarrow$  (+) or (-) mating types. In a number of the earlier crosses, however, nectar transfers were made from a single haploid pustule of one variety to several haploid pustules of another, i.e., (+) or (-)  $\rightarrow$  (+) or (-) mating types. To render such crosses comparable with the others, it was necessary, in the preparation of Table I, to divide by two the total number of the nectar-receiving pustules of these crosses. In the table, the crosses are, therefore, reported as if mass transfers of nectar (containing pycniospores of both (+) and (-) mating types) had been used in every case.

In the crosses, the varieties of *P. graminis* were paired in all possible combinations, that is, crosses and reciprocal crosses were made between each pair of varieties. Some of these varietal combinations were not made in sufficient number to permit a conclusive estimation of intervarietal fertility and, in general, the figures given in the last column of Table I should not be regarded as absolute values but merely as indications of fertility between the different varieties.

In connection with these crosses, two possible sources of error should be mentioned. One was the occurrence of a certain amount of fortuitous selfing, that is, the occasional, apparently spontaneous production of aecia in presumably haploid pustules that had received a transfer of nectar from another variety of the rust. Aecial pustules that apparently arose by selfing are not included in Table I. It is nevertheless possible that some instances of apparent selfing were in reality crosses in which the "female" parent race showed complete dominance. But, as progeny studies with a few cases suspected of showing complete dominance demonstrated that these were



TABLE I  
FERTILITY IN CROSSES BETWEEN VARIETIES OF *Puccinia graminis*

Varieties crossed	No. pustules to which nectar applied	No. pustules that formed aecia	No. pustules of aecia that produced no infection	No. pustules of aecia regarded as hybrid	Probable percentage fertility
<i>Tritici</i> × <i>Avenae</i>	122	23	9	14	11-19
<i>Avenae</i> × <i>Tritici</i>	86	2	2	0	0- 2
<i>Tritici</i> × <i>Secalis</i>	17	14	0	14	82
<i>Secalis</i> × <i>Tritici</i>	7	4	4	0	0-57
<i>Tritici</i> × <i>Agrostidis</i>	34	16	1	15	44-47
<i>Agrostidis</i> × <i>Tritici</i>	28	0	0	0	0
<i>Tritici</i> × <i>Poa</i>	38	19	2	17	45-50
<i>Poa</i> × <i>Tritici</i>	12	1	1	0	0- 8
<i>Avenae</i> × <i>Secalis</i>	31	4	0	4	13
<i>Secalis</i> × <i>Avenae</i>	44	14	4	10	23-32
<i>Avenae</i> × <i>Agrostidis</i>	41	23	8	15	37-56
<i>Agrostidis</i> × <i>Avenae</i>	55	29	1	28	51-53
<i>Avenae</i> × <i>Poa</i>	47	28	10	18	39-60
<i>Poa</i> × <i>Avenae</i>	14	9	6	3	21-64
<i>Secalis</i> × <i>Agrostidis</i>	16	7	3	4	25-44
<i>Agrostidis</i> × <i>Secalis</i>	21	0	0	0	0
<i>Secalis</i> × <i>Poa</i>	27	7	3	4	15-26
<i>Poa</i> × <i>Secalis</i>	49	2	2	0	0- 4
<i>Agrostidis</i> × <i>Poa</i>	38	36	11	25	66-95
<i>Poa</i> × <i>Agrostidis</i>	23	20	9	11	48-87

Note: In the above crosses, nectar was transferred from many pycnial pustules of the last-mentioned variety to individual, and presumably haploid pustules of the first-mentioned. If interfertility of any two varieties were complete, all of the nectar-receiving pustules would produce aecia, and fertility (last column) would be recorded as 100%.

instances of selfing, it is unlikely that dominance was a factor of any appreciable importance in any of the crosses except possibly those between varieties *Poa* and *Agrostidis*. Another possible source of error was the presence, in certain crosses, of aecial pustules from which no uredial cultures were derived. In calculating the percentages of fertility, it was assumed that the aecia in these pustules were of hybrid origin. Where a range is given as (*Tritici* × *Avenae*, 11 to 19%) the first figure represents interfertility based on the number of aecial pustules of known hybridity, whereas the second figure is based on aecial pustules of known hybridity as well as those from which no uredial cultures were established.

The results of the crossing studies make it evident that the degree of fertility between varieties of the rust varies considerably, depending on which varieties are crossed. The crosses between varieties *Tritici* and *Secalis*, *Avenae* and

*Agrostidis*, *Avenae* and *Poae*, and *Agrostidis* and *Poae* were relatively high in fertility. Much lower in fertility were the crosses between varieties *Tritici* and *Avenae*, *Secalis* and *Avenae*, and *Secalis* and *Poae*.

In crosses between certain varieties, the degree of fertility varied according to the direction of the crosses. *Tritici* × *Agrostidis* crosses (*Tritici* pustules receiving *Agrostidis* nectar) indicated fertility of about 45%. The reciprocal crosses indicated complete sterility. Similarly *Tritici* × *Avenae*, *Tritici* × *Poae*, *Secalis* × *Agrostidis*, and *Secalis* × *Poae* crosses succeeded much better than the reverse crosses. The only varieties showing a high degree of interfertility, irrespective of the direction of nectar transfer, were varieties *Agrostidis* and *Poae*. A moderate interfertility seemed also to exist between these varieties and variety *Avenae* regardless of the direction of the transfer of nectar.

In all cases in which any considerable degree of sterility existed between varieties, the aecia formed were fewer in number and smaller in size than in those cases in which fertility was high. The failure to establish uredial cultures from a number of aecial pustules was largely due to the scantiness of aecial material resulting from these causes. Moreover, there appeared to be a relationship between rate of aecial development and degree of sterility between varieties—the greater the sterility the slower the development of the aecia. Where fertility was high, as in crosses between varieties *Agrostidis* and *Poae*, the rate of aecial formation was similar to that prevailing in selfing studies or in crosses involving the races of a single variety, the aecia breaking through the epidermis in six or seven days after the intermixing of nectar. In crosses between varieties *Avenae* and *Agrostidis*, in which sterility was moderate, aecia formed within a period of 7 to 13 days. In *Secalis* × *Agrostidis* crosses the period was 11 to 13 days and in *Tritici* × *Poae* crosses 9 to 16 days.

The variation that exists in the interfertility of the different varieties of *P. graminis* may possibly be interpreted as throwing some light on the relationship between varieties. Varieties *Tritici* and *Secalis* are evidently closer to each other genetically than either is to variety *Avenae*. The grass-inhabiting varieties *Agrostidis* and *Poae* seem to be closely related; and variety *Avenae* is probably more closely related to these than to the other two cereal-inhabiting rusts.

### Pathogenic Characteristics of Hybrid Strains

In all crosses that resulted in the production of aecia, attempts were made to establish uredial cultures by inoculating hosts congenial to the two parent varieties used in a cross as, for instance, wheat and oats in the case of *Tritici-Avenae* crosses. If uredial cultures could be established, further infection tests were made with other cereals and, occasionally, with certain grasses. In these studies, the chief objective was to compare the host range and intensity of the pathogenicity of the hybrid rust with those of the parent rusts used in the cross. In Table II are summarized the pathogenic characteristics of a few of the hybrid rust strains as expressed in infections on wheat, oats, barley, and rye.

TABLE II  
TYPES OF INFECTION PRODUCED BY INTERVARIETAL HYBRID RUSTS ON CEREAL VARIETIES AND CERTAIN GRASSES

Host variety	Tritici × Avenae		Tritici × Secalis		Tritici × Agrostidis		Tritici × Poae	Secalis × Avenae		Avenae × Secalis	
	Tr. race 36 × Av. race 11	Tr. race 39 × Tr. race 11	Tr. race 1 × Sec.	Tr. race 34 × Sec.	Tr. race 17 × Agrost.	Tr. race 169 × Agrost.		Sec. × Av. race 11	Sec. × Av. race 8	Av. race 8 × Sec.	Av. race 8 × Sec.
Wheat	0;	0;	x	x	x	x	λ	0;	0	0	1
Little Club	0;	0;	0;	0;	0	0;	0;	0;	0	0	0;
Marquis	0;	2-	0	0	0	1-	x-	0;	0	0	0;
Kanred	0;	0;	0;	0;	0	0;	0	0;	0	0	0;
Kota	0;	0;	0;	0;	0	0;	0	0;	0	0	0;
Arnaulka	0;	0;	0;	0;	1-	0;	0	0;	0	0	0;
Mindum	0;	0;	0;	0;	1	1-	0	0	0	0	0;
Spelmars	0;	0;	0;	0;	0	0;	0	0;	0	0	0;
Kubanka	0;	0;	0;	0;	0	0;	0	0	0	0	0;
Acme	0;	0;	0;	0;	0	0;	0	0;	0	0	0;
Einkorn	x-	1	x	0;	1 to 1	1-	0	1	0	0	0;
Vernal	0;	0;	0;	0;	0	0;	0	0	0	0	0;
Khapli	0;	0;	0;	1-	0;	1=	0;	0	0	0	0;
Oats	x	x	0	0	0	0;	0	x	x	x	0
Victory	2 to x	2 to x	0	0	0	0	0	x	2±	x	0
White Russian	x	1 to x	0	0	0	0	0	x	2 to x	x	0
Richland	2 to x	2 to x	0	0	0	0	0	x	2 to x	x	0
Sevnothree	2 to x	2 to x	2 to x	2±	2 to 3	3	1 to 2	x	1 to x	x	1+
Barley	2 to x	1	0;	1-	0;	0;	1 to 2	x	x	x	x=
Rye	1 to 2	1	0;	0	0 to 2	1-	0	x	1+	1+	1+
Agrostis alba	-	-	-	-	-	0	0	-	-	-	-
Poa compressa	-	-	-	-	-	0	1 to 3	-	-	-	-
Poa amphila	-	-	-	-	-	0	1 to 3	-	-	-	-

Explanation of symbols: 0 - immunity (; following 0 indicates presence of small necrotic flecks); 1 - high resistance; 2 - moderate resistance; 3 - moderate susceptibility; 4 - complete susceptibility; x - indeterminate reaction; (+) (+ +) (-) (=) - indicate quantitative variations in the infection type.

In general, it was found that hybrid cultures derived from different crosses between the same two varieties showed marked similarity in pathogenic properties. Some variation, however, did occur. For example, one of the three hybrid cultures from the crosses between *Triticum* race 36 and *Avenae* race 11 produced only necrotic flecks on Kanred wheat while the two other cultures produced a 2 type of infection. Though somewhat similar variation occurred in some other related crosses, it was rarely of such magnitude as to dispel the impression of pathogenic resemblance in cultures of similar origin.

In nearly all of the hybrid cultures there was a marked tendency for the hybrid rust to possess a wider host range than did either of the parental varieties. The extension of the host range was, however, accomplished at the expense of pathogenicity—for in no case was the pathogenicity of a hybrid rust on a given host as great as that of the parental variety that attacked that host.

*Triticum-Avenae* hybrid cultures combined to some degree the pathogenic powers of both parental varieties, resembling oat stem rust in moderate ability to attack oats, and wheat stem rust in slight ability to attack certain wheat varieties and barley. Despite the fact that the cultures arose from the *Triticum* side of the crosses, they resembled variety *Avenae* more than variety *Triticum*. Pathogenically, these hybrids bore a rather close resemblance to the *Triticum-Avenae* hybrid described previously by Johnson and Newton (5).

*Triticum-Secalis* cultures showed only a very limited pathogenicity towards wheat and rye and none at all towards oats. Barley, which is a host of both parental varieties, was attacked rather lightly. These cultures resembled rather closely certain of the *Triticum-Secalis* hybrids previously described (4).

*Triticum-Agrostidis* cultures showed limited ability to attack barley, wheat, and *Agrostis alba* but were not pathogenic to oats or rye. The general resemblance of these cultures to the hybrid described previously by Johnson, Newton, and Brown (4) is very close.

Most *Triticum-Poa* hybrids were virtually unable to attack wheat. The one recorded in Table II was an exception in that it produced a weak x type infection on Little Club and Kanred. Barley and rye were highly to moderately resistant. *Poa compressa*, a susceptible host of variety *Poa*, was immune but *P. ampla* varied in reaction from resistant to moderately susceptible.

*Avenae-Secalis* hybrids also were virtually nonpathogenic to wheat but showed some resemblance to both parental varieties by slight to moderate ability to attack oats and rye. They also possessed a limited ability to attack barley.

Hybrids between varieties *Avenae* and *Agrostidis* showed only slight ability to attack either oats or *Agrostis alba*. Pustules on oats were for the most part type 1 but a few cultures produced a weak type x. Pustules on *A. alba* never exceeded type 1.

Most *Avenae-Poae* hybrids appeared to be nearly nonpathogenic to both oats and *Poa compressa* and failed, uniformly, to attack wheat, rye, or *Agrostis alba*. One hybrid proved an exception in that it was capable of producing an x type infection on the oat varieties Victory and White Tartar. It was, however, unable to attack either *Poa compressa* or *P. ampla* and was non-pathogenic to wheat and rye.

Only scanty information is available on the pathogenicity of *Secalis-Agrostidis* hybrids. Rye and *Agrostis alba* appeared to be immune and barley highly resistant.

Varieties *Secalis* and *Poae* do not cross readily. In the four successful crosses, in which the hybrid aecia arose from *Secalis* haplonts that had received *Poae* pycniospores, rye was susceptible and *Poa compressa* moderately resistant. *P. pratensis* and *Agrostis alba* were immune as were also wheat and oats. In these crosses, the pathogenicity of the *Secalis* parent appeared to be inherited intact and to this was added a limited ability to infect *Poa compressa*.

Hybrids between varieties *Agrostidis* and *Poae* were uniformly unable to attack *Agrostis alba* but exhibited on *Poa compressa* a pathogenicity ranging from necrotic flecks and 1 type pustules to 3 type pustules—in terms of varietal reaction a range from high resistance to moderate susceptibility. *Poae* pathogenicity was therefore largely dominant to that of *Agrostidis*. Only a few of the hybrids showed any ability to attack *Poa pratensis*, and in this respect they again resembled the *Poae* parent rust, which was generally incapable of attacking that species. All of the hybrid rust cultures tested on *Poa ampla* attacked this host as would be expected since it is fully susceptible to the *Poae* variety and at least moderately so to the *Agrostidis* variety. Wheat, oats, and rye were not visibly attacked by any of the hybrids.

### Inheritance of Urediospore Size and Color

The classification of *Puccinia graminis* into varieties is based on host specificity rather than on the morphology of the spores. Nevertheless, as has been clearly established by Levine (7) and Stakman and Levine (9), there are considerable differences in size and shape between the spores of certain of the varieties. The urediospores of the cereal-inhabiting varieties *Tritici* and *Avenae*, are distinctly larger than those of the grass-inhabiting varieties *Agrostidis* and *Poae* while the spores of variety *Secalis* occupy a somewhat intermediate place.

Owing to the similarity in size of the urediospores of varieties *Tritici*, *Avenae*, and *Secalis*, no attempt was made to study the inheritance of spore size in crosses between them. When, however, crosses were successfully made between any of these varieties and the smaller-spored varieties *Agrostidis* and *Poae*, comparative measurements were usually made of 10 or 20 urediospores of the hybrid and a similar number of spores of the two parent rusts.

Whenever possible the hybrid spores were compared with the actual parent strains used in the cross rather than with other accessions of the parent rust varieties.

The data thus acquired were summarized (Tables III and IV) and submitted to statistical analysis on the assumption that the combined data from crosses between any two varieties might permit generalization as to whether or not the urediospores of the hybrids were significantly different in size or shape from those of the parent rusts.

The results show that in the crosses *Tritici-Poae*, *Secalis-Poae*, *Avenae-Poae*, and *Avenae-Agrostidis* the hybrid spores are significantly different in both length and width from those of both parent varieties. Only in the *Agrostidis-Poae* crosses was there no significant size difference between hybrids and parents. Where differences existed, they were always manifested by a spore size roughly intermediate between those of the parent rusts.

In general, the hybrid urediospores did not exhibit any noticeable abnormality in form or in spore contents, but occasional hybrids showed some deviation from the normal. One cross of *Avenae* race 1  $\times$  *Poae* produced urediospores very irregular in shape and size and in organization of the spore contents. The orange pigment, which normally is present in an oval granular mass in the center of the spore, was gathered into scattered oil-like droplets or was entirely absent. In another cross of the same origin, the orange pigment was commonly gathered together into one large oil-like globule in the center of the spore.

In crosses in which the urediospore color of one parent differed from the normal, red color of stem rust, there was a strong tendency for the red color to predominate in the hybrid spores. The cross *Secalis*  $\times$  *Avenae* race 4 orange produced a hybrid pathogenically much closer to the *Avenae* parent rust but with the red spore color of the *Secalis* parent. The cross *Avenae* race 6 orange  $\times$  *Poae* produced urediospores that showed much of the red color of the *Poae* parent when viewed in mass. When examined with the microscope, it was seen that many spores were irregular in shape and content, some being nearly hyaline with the pigment concentrated in bright yellow droplets. Pathogenically this rust resembled the *Avenae* more than the *Poae* parent.

In one cross, *Agrostidis*  $\times$  *Avenae* race 2 grayish-brown, the hybrid spores were of a color intermediate between the bright red of the *Agrostidis* rust and the dull grayish-brown of the *Avenae* parent. When viewed with the microscope, it was evident that the hybrid spores contained less orange pigment than the *Agrostidis* spores but more than those of the *Avenae* parent race.

It seems clear from the above studies that intervarietal hybrid rusts show a definite tendency to produce urediospores intermediate between those of the parent varieties in size and shape. Observable color differences between

TABLE III

FREQUENCY DISTRIBUTIONS FOR LENGTH AND WIDTH OF UREDIOSPORES OF VARIETIES OF *P. graminis* AND OF CERTAIN INTERVARIETAL HYBRIDS, WITH RELEVANT STATISTICS

Varieties	Spore classes according to:										Total no. spores	Statistics				
	Length ( $\mu$ )											Mean and S.e. of mean	Standard deviation and S.e. of standard deviation	Coefficient of variability and S.e. of V.		
	15	18	21	24	27	30	33	36	39							
<i>Tritic</i>					6	3		4	1	20	31.65 $\pm$ 0.83	3.73 $\pm$ 0.59	11.79 $\pm$ 1.89			
<i>Avenae</i>				11	15	21		2		60	28.90 $\pm$ 0.42	3.24 $\pm$ 0.30	11.21 $\pm$ 1.04			
<i>Secalis</i>			2	12	3	2		1		20	25.35 $\pm$ 0.75	3.35 $\pm$ 0.53	13.21 $\pm$ 2.13			
<i>Agrostidis</i>	2	1	16	23	8	2				50	23.64 $\pm$ 0.36	2.52 $\pm$ 0.25	10.66 $\pm$ 1.08			
<i>Poa</i>		20	29	16	3					70	20.91 $\pm$ 0.32	2.68 $\pm$ 0.23	12.82 $\pm$ 1.10			
<i>Tritic</i> $\times$ <i>Poa</i>		1	6	9	8	6				30	25.20 $\pm$ 0.61	3.34 $\pm$ 0.43	13.25 $\pm$ 1.74			
<i>Avenae</i> $\times$ <i>Agrostidis</i>			4	23	14	7				50	25.80 $\pm$ 0.41	2.88 $\pm$ 0.29	11.16 $\pm$ 1.13			
<i>Avenae</i> $\times$ <i>Poa</i>	1	7	16	19	25	16		1		90	25.43 $\pm$ 0.45	4.27 $\pm$ 0.32	16.79 $\pm$ 1.29			
<i>Secalis</i> $\times$ <i>Poa</i>		4	17	11	6		2			40	23.02 $\pm$ 0.54	3.45 $\pm$ 0.39	14.99 $\pm$ 1.70			
<i>Agrostidis</i> $\times$ <i>Poa</i>			7	9	3	1				20	23.70 $\pm$ 0.56	2.50 $\pm$ 0.40	10.55 $\pm$ 1.69			
	Width ( $\mu$ )															
<i>Tritic</i>	12	13	14	15	16	17	18	19	20	21	22		20	20.10 $\pm$ 0.22	0.97 $\pm$ 0.15	4.83 $\pm$ 0.76
<i>Avenae</i>				1	2	3	12	1	1	6			60	19.17 $\pm$ 0.17	1.30 $\pm$ 0.12	6.68 $\pm$ 0.61
<i>Secalis</i>			1	1	8	5	1	4	8	9			20	17.30 $\pm$ 0.30	1.36 $\pm$ 0.22	7.86 $\pm$ 1.24
<i>Agrostidis</i>			4	18	27			1					50	16.04 $\pm$ 0.11	0.80 $\pm$ 0.08	4.99 $\pm$ 0.50
<i>Poa</i>			33	26	11								70	15.19 $\pm$ 0.09	0.73 $\pm$ 0.06	4.81 $\pm$ 0.41
<i>Tritic</i> $\times$ <i>Poa</i>			1	8	16	2	3						30	16.43 $\pm$ 0.17	0.93 $\pm$ 0.12	5.66 $\pm$ 0.73
<i>Avenae</i> $\times$ <i>Agrostidis</i>			1	2	24	10	6	7					50	17.28 $\pm$ 0.17	1.20 $\pm$ 0.12	6.94 $\pm$ 0.69
<i>Avenae</i> $\times$ <i>Poa</i>	1	1	7	8	22	4	9	25	4		5		90	17.99 $\pm$ 0.24	2.29 $\pm$ 0.17	12.73 $\pm$ 0.97
<i>Secalis</i> $\times$ <i>Poa</i>			3	11	21	5							40	16.20 $\pm$ 0.12	0.78 $\pm$ 0.09	4.81 $\pm$ 0.54
<i>Agrostidis</i> $\times$ <i>Poa</i>		1	7	6	6								20	15.35 $\pm$ 0.20	0.91 $\pm$ 0.14	5.93 $\pm$ 0.94

TABLE IV

DIFFERENCES (IN MICRONS) IN MEAN SPORE LENGTH AND MEAN SPORE WIDTH OF UREDIOSPORES OF VARIETIES OF *P. graminis* AND OF CERTAIN INTERVARIETAL HYBRIDS. (THE FIGURES ABOVE THE DIAGONAL LINE REPRESENT DIFFERENCES IN MEAN SPORE LENGTH, THOSE BELOW SHOW DIFFERENCES IN MEAN SPORE WIDTH)

	<i>Tritici</i>	<i>Avenae</i>	<i>Secalis</i>	<i>Agrostidis</i>	<i>Poae</i>	<i>Tritici</i> × <i>Poae</i>	<i>Avenae</i> × <i>Agrostidis</i>	<i>Avenae</i> × <i>Poae</i>	<i>Secalis</i> × <i>Poae</i>	<i>Agrostidis</i> × <i>Poae</i>
Differences in mean spore length										
<i>Tritici</i>		** 2.75	** 6.30	** 8.01	** 10.74	** 6.45	** 5.85	** 6.22	** 8.63	** 7.95
<i>Avenae</i>	* 0.63		** 3.55	** 5.26	** 7.99	** 3.70	** 3.10	** 3.47	** 5.88	** 5.20
<i>Secalis</i>	** 2.80	** 2.17		* 1.71	** 4.44	0.15	0.45	0.08	* 2.33	1.65
<i>Agrostidis</i>	** 4.06	** 3.43	** 1.26		** 2.73	* 1.56	** 2.16	** 1.79	0.62	0.06
<i>Poae</i>	** 4.91	** 4.28	** 2.11	** 0.85		** 4.29	** 4.89	** 4.52	** 2.11	** 2.79
<i>Tritici</i> × <i>Poae</i>	** 3.67	** 3.04	* 0.87	0.39	1.24		0.60	0.23	** 2.18	1.50
<i>Avenae</i> × <i>Agrostidis</i>	** 2.82	** 2.19	0.02	1.24	2.09	0.85		0.37	** 2.78	** 2.10
<i>Avenae</i> × <i>Poae</i>	** 2.11	** 1.48	0.69	1.95	2.80	1.56	* 0.71		** 2.41	* 1.73
<i>Secalis</i> × <i>Poae</i>	** 3.90	** 3.27	** 1.10	0.16	1.01	0.23	** 1.08	** 1.79		0.68
<i>Agrostidis</i> × <i>Poae</i>	** 4.75	** 4.12	** 1.95	** 0.69	0.16	** 1.08	** 1.93	** 2.64	** 0.85	
Differences in mean spore width										

\* = Difference significant at 5% point.

\*\* = " " " 1% " .

varieties do not normally exist, but when they do there is a strong tendency to a complete or nearly complete dominance of normal color characteristics in the hybrid.

### Progeny Studies with Intervarietal Hybrids

It has been shown (6) that, in the selfing of hybrids between two physiologic races of the variety *Tritici*, the next generation ( $F_2$ ) will contain the pathogenic characters of the grandparental races in combinations that give rise to many physiologic races. Among these, both grandparental races are likely to recur.



In such crosses, the pathogenic properties appear to be transmitted as unit characters that are reshuffled to form new combinations.

If the same pattern of behavior prevailed in intervarietal crosses, it might be expected that in *Triticum-Avenae* crosses, for example, the  $F_2$  generation would contain strains that combined in various ways the pathogenic properties of both rusts. Some strains might attack certain wheat and oat varieties heavily, whereas others might be predominantly wheat or oat strains. Possibly, even one or both of the grandparental races might occur in the  $F_2$  generation.

Such selfing studies as have been carried out with intervarietal hybrids show a behavior differing considerably from that described above.

Two crosses of var. *Triticum* race 36  $\times$  var. *Avenae* race 11 produced practically identical results (See Table V). The  $F_1$  hybrid rust combined to some degree the pathogenic powers of both parent rusts. It resembled oat stem

TABLE V

SUMMARY OF STUDIES ON THE SELFING OF INTERVARIETAL HYBRID RUSTS

Varieties crossed	Pathogenicity in $F_1$ uredial generation	Symptoms on barberry inoculated with $F_1$ teliospores	Pathogenicity in $F_2$ uredial generation
<i>Triticum</i> race 36 $\times$ <i>Avenae</i> race 11	Moderately pathogenic to oats and barley; slightly to wheat and rye	Small pycnia with scanty nectar. No aecia. Uredia and telia formed occasionally on old infections	Urediospores formed on barberry produced weak infection on oats; no infection on wheat
<i>Triticum</i> race 36 $\times$ <i>Avenae</i> race 11	As above	As above	Urediospores formed on barberry failed to cause infection
<i>Secalis</i> $\times$ <i>Avenae</i> race 11	Moderately pathogenic to oats, rye, and barley. Almost nonpathogenic to wheat	Small pycnia with scanty nectar. No aecia. Uredia and telia formed in one old infection	Oats highly to moderately resistant. Barley and rye highly resistant. Wheat nearly immune
<i>Triticum</i> race 95 $\times$ <i>Secalis</i>	$F_1$ identified as <i>Triticum</i> race 104, moderately pathogenic to barley and slightly so to wheat and rye	Normal pycnia and aecia	The few tests made indicated that wheat and barley were moderately resistant and rye nearly immune
<i>Triticum</i> race 30 $\times$ <i>Secalis</i>	$F_1$ identified as <i>Triticum</i> race 70, moderately pathogenic to barley and slightly so to wheat and rye	Normal pycnia and aecia	The following races of var. <i>Triticum</i> identified (number of times each identified, in brackets): 71 (4); 104 (3); 111 (1); 137 (4); 138 (7); 153 (2)
<i>Triticum</i> race 1 $\times$ <i>Secalis</i>	$F_1$ identified as <i>Triticum</i> race 103, moderately pathogenic to barley and slightly so to wheat and rye. Nonpathogenic to oats	Normal pycnia and aecia	Var. <i>Triticum</i> races 71 (1); 103 (34); 111 (10); 112 (2). All of the numbered races in the two above crosses are moderately pathogenic to barley and slightly pathogenic to wheat and rye

rust in moderate ability to attack oats, *Dactylis glomerata*, *Arrhenatherum elatius*, and *Lolium perenne*. It resembled wheat stem rust in slight ability to attack certain wheat varieties and barley. Despite the fact that the rust originated from the *Tritici* side of the cross, it resembled oat stem rust in pathogenicity more than wheat stem rust.

Selfing studies with the two hybrid rusts produced identical results. On barberry, both produced small pustules of pycnia with only a trace of nectar in which, however, a considerable number of pycniospores were present. No aecia were formed in consequence of intermixing this nectar, but urediospores and teliospores occurred in a few of these pustules between two and three months after infection.

In several attempts to produce infection on cereals with the urediospores just mentioned, only one positive result was obtained—a weak infection of Victory oats. The urediospores differed in several respects from those of normal oat stem rust: they were less regular in shape and size, with many shrunken, colorless spores as well as a high proportion of spores containing one or more yellow, oil-like droplets in the center of the spore instead of the granular yellow mass characteristic of normal rust spores.

It seems probable that the pycniospores produced by the *Tritici-Avenae* hybrid are functionless as the application of the scanty pycnial nectar to haploid pustules of var. *Avenae* race 11 failed to produce aecia in these pustules. The failure of the hybrid race itself to produce aecia in repeated selfings may not be solely due to lack of function on the part of the pycniospores as liberal applications of nectar of var. *Avenae* races 1 and 12 and of var. *Tritici* race 15 to the pycnia of the hybrid likewise failed to produce aecia.

Very similar results were secured in the selfing of the  $F_1$  hybrid of the cross var. *Secalis*  $\times$  var. *Avenae* race 11. The hybrid rust, which arose from the *Secalis* side of the cross, had some of the pathogenic powers of both parent rusts but resembled oat stem rust more than rye stem rust. Numerous infections were obtained on barberry with teliospores formed on oats and barley. The pycnia produced scarcely any nectar and no aecia resulted from the intermixing of such nectar as was present. Uredia, and subsequently telia, were formed in one infection about two months after inoculation. The urediospores infected Victory oats lightly (x type infection) and other oat varieties still more lightly. Barley was moderately to highly resistant; rye highly resistant; and wheat virtually immune.

The selfing of  $F_1$  hybrids of crosses between varieties *Tritici* and *Secalis* has produced notably different results. The hybrids that have been studied have produced normal pycnia and aecia on barberry and no indications of sterility have been noted.

On cereals, the pathogenicity of the progeny of the three *Tritici*  $\times$  *Secalis* crosses studied has been rather strikingly similar. In each case, the  $F_1$  uredial generation was moderately pathogenic to barley and, in general, only slightly pathogenic to wheat and rye.

It might have been expected that in the  $F_2$  uredial generation (resulting from the selfing of the  $F_1$  hybrid) some rather typical wheat stem rust and rye stem rust would have appeared as well as strains similar to those that comprised the  $F_1$  generation. Actually, the pathogenic variation in  $F_2$  was rather narrowly limited to strains of rust that bore a close resemblance to the  $F_1$  generation, and no pathogenically vigorous strains of either wheat stem rust or rye stem rust could be discovered.

The results in the  $F_2$  generation of the cross *Triticum* race 1  $\times$  *Secalis* are typical of the crosses that have been studied in the  $F_2$  generation (Table VI).

TABLE VI

PATHOGENICITY ON WHEAT, BARLEY, AND RYE OF RACES OCCURRING IN  $F_1$  AND  $F_2$  GENERATIONS OF THE CROSS VAR. *Triticum* RACE 1  $\times$  VAR. *Secalis*

Variety tested	Parent races		$F_1$	Physiologic races in $F_2$			
	<i>Triticum</i> race 1	<i>Secalis</i>	Race 103	71	103	111	112
Little Club	4	0;	x+	3+	x+	3±	4
Marquis	4-	0	0;	x-	0	1-	x
Kanred	0	0	0	0	0;	0	0
Kota	3+	0	0;	0;	0	0;	0;
Arnautka	1=	0	0;	0;	0	0;	0;
Mindum	1	0	0;	1=	0;	0;	0;
Spelmars	1=	0	0;	0;	0;	0;	0;
Kubanka	3+	0	0;	0;	0;	0;	x-
Acme	3+	0	0	0;	0;	0;	0;
Einkorn	3	0	2-	1	2-	1-	3
Vernal	0;	0	0;	0;	1=	0;	0;
Khapli	1-	0	0;	0;	0;	1-	1-
Barley vars.	3-	3-	2-3	1-3	2-3	2-3	3
Rye vars.	0;	4	1-3	0;	1-3	1-3	1

*Explanation of symbols:*

0 - immunity (; following 0 indicates presence of small necrotic flecks)

1 - high resistance

2 - moderate resistance

3 - moderate susceptibility

4 - susceptibility

x - indeterminate reaction

(+) (++) (-) (=) - indicate quantitative variations in the infection type

All cultures derived from the aeciospores were very similar irrespective of whether they were established on wheat or rye. Though identifiable as different physiologic races, they were either pathogenically identical with the  $F_1$  hybrid, which had been classified as race 103 of var. *Triticum*, or they differed only slightly from it. The cultures established on Little Club wheat were identified as race 103 (15 cultures); race 111 (6 cultures); and race 112 (1 culture). Those established on Rosen rye were identified as race 71 (1 culture); race 103 (9 cultures); race 111 (4 cultures); and race 112 (1 culture).

These races, which occur rarely if at all in field collections, had all been found previously in the  $F_1$  generation of *Tritici-Secalis* crosses and may therefore be regarded as characteristic progeny of such crosses.

Although the races shown in Tables VI and VII are all assigned to var. *Tritici*, the propriety of doing so is rather doubtful as, with the exception of

TABLE VII

PATHOGENICITY ON WHEAT, BARLEY, AND RYE OF RACES OCCURRING IN  $F_1$  AND  $F_2$  GENERATIONS OF THE CROSS VAR. *Tritici* RACE 30  $\times$  VAR. *Secalis*

Variety tested	Parent races		$F_1$ Race 70	Physiologic races in $F_2$					
	<i>Tritici</i> race 30	<i>Secalis</i>		71	104	111	137	138	153
Little Club	4	0;	3+	3	4	3±	4	2	3±
Marquis	4	0	x+	x-	x=	1-	4-	1-	1+
Kanred	0	0	0	0	0	0	4-	0	3-
Kota	3+	0	0;	0;	0;	0;	0;	0;	0;
Arnautka	x	0	0;	0;	0;	0;	1-	0;	0;
Mindum	x	0	0;	1=	0;	0;	1-	0;	0
Spelmars	x	0	0;	0;	0;	0;	1-	0;	0
Kubanka	x	0	0;	0;	0;	0;	3	0;	0;
Acme	x	0	0;	0;	0;	0;	1-	0;	0
Einkorn	3+	0	1=	1	3+	1-	3-	1+	0;
Vernal	4=	0	x	0;	x+	0;	x-	x-	x-
Khapli	1	0	1=	0;	0;	1-	1	0;	0;
Barley vars.	3	3-	3	1-3	1-3	2-3	2-3	2-3	-
Rye vars.	0;	4	1	0;	1-3	1-3	1-3	1-3	-

race 137, their pathogenicity to wheat varieties is extremely limited. None of the 12 differential hosts is fully susceptible to race 103 or race 111; only one variety (Little Club) is fully susceptible to race 71; and only two (Little Club and Einkorn) are susceptible to race 112. Many other wheats susceptible to most physiologic races of var. *Tritici* were tested and found to be moderately or highly resistant to these races. The pathogenicity of these races towards rye is even more limited as the variety most severely attacked (Rosen) cannot be regarded as fully susceptible. Barley, which is a common host of both the parental rusts used in the cross (var. *Tritici* and var. *Secalis*), is probably a more congenial host to the races occurring in the  $F_1$  and  $F_2$  generations of this cross than either wheat or rye. The ability of these races to attack barley and their limited pathogenicity towards wheat and rye may probably be interpreted as proof that the hybrid progeny is pathogenically intermediate between wheat stem rust and rye stem rust.

The absence, in the  $F_2$  generation of *Tritici-Secalis* crosses, of the grand-parental wheat stem rust races or of typical rye stem rust does not necessarily prove that they never occur as segregants from similar crosses. That rather typical *Tritici* races may occur in  $F_2$  is suggested by the presence in the  $F_2$  generation of the cross *Tritici* race 30  $\times$  *Secalis* of race 137, which has a

sufficiently wide pathogenic range on wheat varieties to be accepted as a fairly typical wheat stem rust race (Table VII). The rarity of typical *Tritici* strains and the absence of *Secalis* strains in the  $F_2$  generations of the crosses studied does, however, suggest the presence of some condition that prevents their appearance. Certain characteristics of the *Tritici* races used in the crosses failed completely to appear in the  $F_2$  generation. For example, all three of the *Tritici* parent races (races 1, 30, and 95) were highly pathogenic to the wheat variety Acme. None of the  $F_2$  progeny showed any appreciable degree of pathogenicity on this variety. The moderate ability of race 30 to attack the durum wheats Arnautka, Mindum, Spelmars, and Kubanka did not appear at all in the  $F_2$  generation of the cross *Tritici* race 30  $\times$  *Secalis*. On the other hand, there occurred in the  $F_2$  generation of this cross a wheat stem rust characteristic not shown by the parent *Tritici* race. Race 30, the *Tritici* parent, does not attack the wheat variety Kanred. The  $F_2$  generation, however, contained race 137, which attacks Kanred vigorously, and race 153, which attacks it moderately. Presumably, the occurrence in  $F_2$  of ability to attack Kanred is due to heterozygosity of the race 30 parent for this character, which is known to behave as a recessive.

The reason for the nonappearance in  $F_2$  of some of the chief pathogenic characteristics of the parental rusts is not at all clear. It is not surprising that strains identical to the wheat stem rust and rye stem rust entering into the crosses should not appear in the relatively small  $F_2$  populations studied. As the genotype of each of the parents is made up of many factors, it is not to be expected that those identical factorial combinations would recur frequently in  $F_2$ . The great majority of the  $F_2$  combinations would be heterozygous for several *Tritici* and *Secalis* factors. Those that approach homozygosity for, say, *Tritici* factors might contain *Secalis* genes or chromosome segments in a homozygous condition that would exclude from the genotype the corresponding *Tritici* genes or segments. A genotype unbalanced or deficient with respect to the *Tritici* complement might result in nonviability, the effect of which would be the infrequent appearance of true *Tritici* types. It is true that such genotypic unbalance might also occur in the more heterozygous combinations but, as these make up the bulk of  $F_2$  combinations, the effect on their frequency of appearance would be less evident.

Another possible explanation involves the relationship of nuclei and cytoplasm. As nuclei and cytoplasm both affect pathogenicity, possibly the reason is to be found in some interaction between the two. It may be that, in hybrids arising from *Tritici* haplonts, the cytoplasm in which the nuclei operate is chiefly of *Tritici* origin. If so, there might be a tendency in such crosses towards favoring the expression of *Tritici* characteristics in both  $F_1$  and  $F_2$  and a corresponding tendency towards partial inhibition of the expression of *Secalis* characteristics. In hybrids arising from *Secalis* haplonts, this tendency would presumably be reversed. Such an assumption would account for the failure of typical rye stem rust to appear in the  $F_1$  and  $F_2$

generations of the crosses in which the hybrids originated from the mycelia of *Tritici* haplonts, but it would not account for the absence of the *Tritici* characteristics mentioned above. Only a comparative study of reciprocal (*Secalis*  $\times$  *Tritici*) crosses could test this idea, and, so far, no such study has been made.

### Discussion

The word "specialization", as applied to a living organism, connotes modification, physiological or morphological or both, in the organism. Regarded from the standpoint of evolution, it implies a divergent development in which different lines of the same organism acquire somewhat different ways or habits of life that eventually become fixed modifications. In *Puccinia graminis*, the adaptation of different lines or clones of the organism to different grass hosts is probably of long standing and has led to the division of the organism into rather narrowly specialized groups. These groups, *formae speciales* or varieties, still maintain contact with each other through their common ability to parasitize the barberry.

If it is granted that the different varieties of *P. graminis* had a common origin, it follows that the species was originally readily capable of forming new binuclear relationships on the barberry. As shown in the present paper, this does not hold true for the species as we know it to-day. Some of the varieties here discussed do not readily combine to form dikaryons. If the establishment of such dikaryons can be regarded as hybridization, then any obstacle to their establishment is to be looked upon as intervarietal sterility, and as interfertility between organisms is a sign of closeness in relationship so is intersterility a sign of divergence. Viewed thus, it is possible to group the varieties on the basis of relationship. *Agrostidis* and *Poa* form one pair of closely related varieties and *Tritici* and *Secalis* form another such pair. In both these pairs, there is confirmatory evidence to be found in their pathogenic behavior. Varieties *Agrostidis* and *Poa* have a common host in *Poa ampla*, and varieties *Tritici* and *Secalis* in barley and a number of grasses. Variety *Avenae* appears to lack close relationship with any of the other four varieties despite the fact that it can form dikaryons with all of them.

Further evidence of the lack of relationship between var. *Avenae* and vars. *Tritici* and *Secalis* is to be found in the behavior of the hybrids on the alternate host, barberry. The hybrid teliospores of *Tritici*  $\times$  *Avenae* and *Secalis*  $\times$  *Avenae* are capable of germination and their sporidia infect barberry readily. That the haploid mycelia grow quite readily is shown by the large size of many of the infections. Sterility, however, results from the fewness of the pycnia formed and their evident lack of ability to produce an abundance of pycnial nectar and possibly also from the failure of the pycniospores to function normally.

The pathogenicity of the  $F_1$  and  $F_2$  hybrids is of interest alike from the practical and the theoretical viewpoint. In any intervarietal hybrid, the  $F_1$  aeciospore and urediospore contains two nuclei, one derived from each parental

variety. Hence it is not unexpected that the hybrid rust should show some of the characteristics of each of the parents. Complete dominance of the pathogenicity of one or the other parent is probably rare but it is not uncommon, as in *Triticum*  $\times$  *Avenae* hybrids, for the hybrid to resemble one rust much more than the other. The factors governing pathogenicity in  $F_1$  are probably mostly nuclear as in *Triticum*  $\times$  *Avenae* crosses the *Avenae* characters predominate despite the fact that the hybrids arose from fertilized *Triticum* haplonts. If the cytoplasm were important in this respect, it would be expected that *Triticum* characters would predominate as the hybrids presumably contain mostly *Triticum* cytoplasm.

In connection with the  $F_2$  generation there is some evidence, derived from *Triticum*  $\times$  *Secalis* crosses, that variation is limited and that the  $F_2$  variants bear a rather close pathogenic resemblance to the  $F_1$ . It may not be safe to generalize from the rather limited studies carried out thus far; but, if this type of variation should be common in intervarietal hybridization, it is clear that it would constitute a factor favoring the creation of new pathogenic types of rust and would be a stabilizing influence tending to perpetuate the type created by the cross.

The results of surveys carried out over many years on the specialization of wheat stem rust, oat stem rust, and, to a certain extent, rye stem rust do not indicate that the creation of new strains of rust through intervarietal hybridization is of frequent occurrence or of much practical importance. These rusts are rather well defined pathogenically and there is rarely any difficulty in assigning any given collection of a rust to one or the other. The work here described indicates that there are two factors that render natural hybridization between the varieties of *P. graminis* of little practical importance. One of these is intervarietal sterility, which militates against the creation of hybrids. The other is the fact that the broadening of the range of pathogenicity, which is so characteristic of intervarietal hybrids, is commonly accompanied by a reduction in pathogenic intensity on any given host.

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## TEMPERATURE AND CULTURAL STUDIES ON *UROCYSTIS TRITICI* KOERN.<sup>1</sup>

BY YU-SAN WU<sup>2</sup>

### Abstract

A study of the effect of temperature on the infection of wheat by *Urocystis tritici* was made using Petri dishes in incubators held at various temperatures from 9° to 26° C. The seedlings were transferred to pots as soon as the plumules broke through the coleoptiles. The percentages of infection obtained at various temperatures were as follows:—10.8% at 9° to 14° C., 13.8 at 16°, 21.1 at 18°, 26.1 at 20°, 15.7 at 22°, 6.9 at 24°, and 3.0% at 26° C. Growth studies of three geographical isolates of *U. tritici* were made on 12 different artificial media. Cultures were obtained by plating bits of disinfected unbroken, fresh, smutted plant tissue. The smut organism was found to be culturable on various media, though its growth rate was rather slow. The three isolates grew well on potato dextrose agar, Thaxter's hard agar, 4% sucrose potato agar, wheat seedling agar, and Czapek's synthetic medium, and were found to differ characteristically on five different media.

### Introduction

Flag smut of wheat is a very important disease in China, especially in the northern wheat growing regions. In general, it causes severe damage to winter wheat, and as a rule is less severe on spring sown wheat. The losses caused by the smut of wheat in 1936 and 1937 were estimated to be 5.0% and 4.0% to 5.0%, respectively.

## Part I. Temperature Studies

### PREVIOUS WORK

Griffiths (6) in 1924 reported that infection occurred at 6° to 12° C., 17° to 19° C., and 21.5° to 23.5° C., but did not occur at 25° to 26.6° C. The highest percentage of infection that she obtained was at 21.5° to 23.5° C. Noble (7) stated in 1924 that the optimum soil temperature for infection of *Urocystis tritici* Koern. ranged from 14° to 21° C. The most severe and, at the same time, the earliest recorded infection occurred on plants kept at 19° to 21° C. Infection also took place at 23° to 25° C., but no infection occurred at 29° to 31° C. In an experiment made by Verwoerd (10) in 1929 infection was secured at 22° C., but no infection occurred at a temperature of 31° C. In 1933, Faris (4) similarly reported that high infection occurred at 10° to 20° C., and also that the optimum temperature could be shifted by the soil moisture content.

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## Material and Methods

Thoroughly mixed sandy loam, obtained from the field, was run through a sieve, and then sterilized by hot air for an hour at a temperature of 160° C. Fifty grams of this dry soil was placed in sterilized Petri dishes. Ten cc. of sterile tap water was added to each dish except in Experiment I, to which 15 cc. of water was added. The seeds were disinfected by soaking in aqueous bleaching powder solution (50% by weight of commercial powder) for 20 min. and washing repeatedly with sterilized water. They were then moistened with a wet cloth so that a large quantity of smut spores would adhere to them. Each Petri dish contained 60 seeds in Experiment I, and 50 in Experiments II, III, and IV. The dishes were then placed at various temperatures in incubators. As soon as the plumules broke through the coleoptiles, the germinating seeds were planted in 8-in. pots, 40 to 50 per pot, at a depth of half an inch below the soil surface as suggested by Angell (1, 2), in order to prevent tillering and to produce symptoms of the disease as early as possible. Temperature fluctuation in each incubator was kept within a range of  $\pm 1^\circ$  C.

The smut was collected from the experiment field in Nanking. A very susceptible variety of wheat, H1102, was used in all experiments. Plants were grown in the greenhouse. The experiment was made in the winter of 1935 and repeated in the winter of 1936. Records were taken when the plants were blossoming.

## Results and Conclusions

The results of the experiment are shown in Table I. The temperature range of infection was from 9° to 26° C., with the optimum at 20° C. The rate of infection was, however, greatly decreased near the two extremes.

TABLE I

THE PERCENTAGE OF INFECTION OF *Urocystis tritici* ON WHEAT UNDER VARIOUS SOIL TEMPERATURES IN 1935 AND 1936

Temperature in degrees centi- grade	Experiments in 1935						Experiments in 1936						Average infection in %
	I			II			III			IV			
	Total plants	Smut- ted plants	Infection in %	Total plants	Smut- ted plants	Infection in %	Total plants	Smut- ted plants	Infection in %	Total plants	Smut- ted plants	Infection in %	
9-14	116	12	10.4	—	—	—	83	11	13.2	93	8	8.6	10.8*
16	—	—	—	—	—	—	84	13	15.5	75	9	12.0	13.8**
18	116	20	17.2	94	3	3.2	89	30	33.5	76	23	30.3	21.1
20	119	31	26.1	97	4	4.1	95	40	42.1	72	23	31.9	26.1
22	102	12	11.8	96	2	2.1	87	21	24.1	65	16	24.6	15.7
24	107	5	4.7	96	3	3.1	96	13	13.5	65	4	6.1	6.9
26	103	3	2.9	96	1	1.1	78	5	6.4	65	1	1.5	3.0

\* The average of three tests.

\*\* The average of two tests.

The results thus obtained are quite similar to those reported by previous workers. It appears that the optimum soil temperature for the infection of flag smut of wheat is approximately 20° C., and probably this is the explanation for winter wheat being more highly infected than spring sown wheat, because, ordinarily, the soil is warmer when the winter wheat is being sown.

## Part II. Cultural Studies

Although many smut fungi have been induced to grow in pure culture, attempts to grow the organism causing flag smut of wheat in pure culture have not hitherto met with success. The first reported attempt to culture *U. tritici* on artificial media was made by Verwoerd (10) in 1929, but was unsuccessful. In 1937 and 1938, the writer tried to culture this fungus, with the results herein reported.

## Materials and Methods

Flag smut isolates were made in 1937 and 1938. The causal organism was isolated in pure culture in 1937 at Nanking from Kaifeng and from Hsueh collections and again in 1938 at Chengtu, Szechuan, from Tsetung collections. The isolation procedure consisted of cutting half centimeter portions of unbroken fresh smutted tissue from smutted plants, soaking these bits of tissue in a 20% solution of commercial bleaching powder or in mercuric chloride solution (1:1000) for 10 min., rinsing in sterile water, and then transferring to culture tubes. Eleven kinds of organic media and one synthetic medium were used. Fifty cc. Erlenmeyer flasks with 25 cc. nutrient media added were used for cultural purposes. These flasks were autoclaved under a pressure of 15 lb. for half an hour. Transfers were made with a loop needle using 37-day-old cultures as sources of inoculum. Each kind of medium was triplicated. After 60 days, the results were recorded.

The description of growth characteristics includes diameter of colony, coloration, topography, consistency, surface, zonation, and margin, following the method used by Christensen and Stakman (3), Rodenhiser (9), and Ficke and Johnston (5) to describe smut growth on media. The nomenclature of coloration was according to Ridgway's *Color standards and color nomenclature* (8).

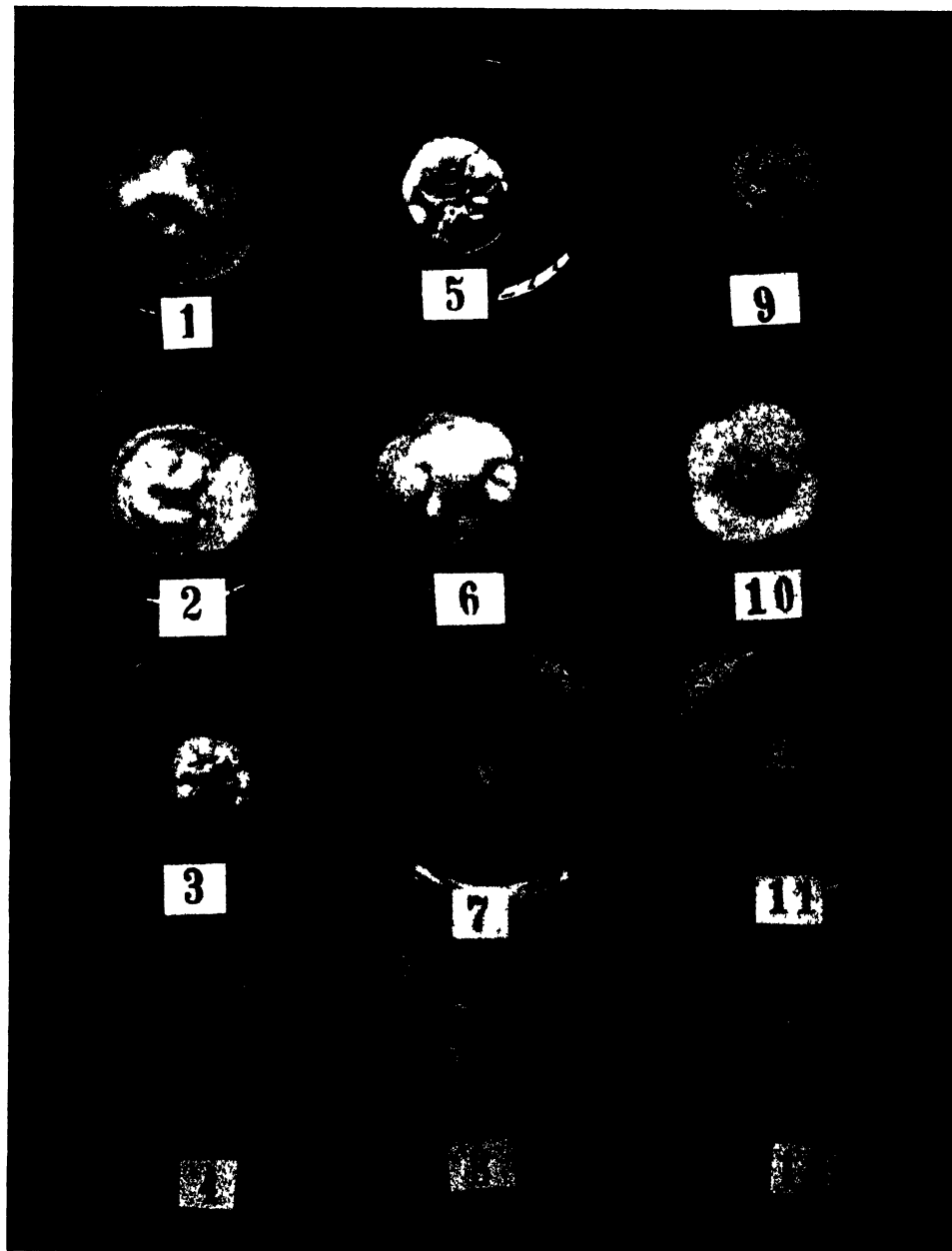
## Results

Cultures from Kaifeng, Hsueh, and Tsetung grew well on the 12 media, especially on potato dextrose agar, Thaxter's hard agar, 4% sucrose potato agar, wheat seedling decoction agar, and Czapek's synthetic medium.

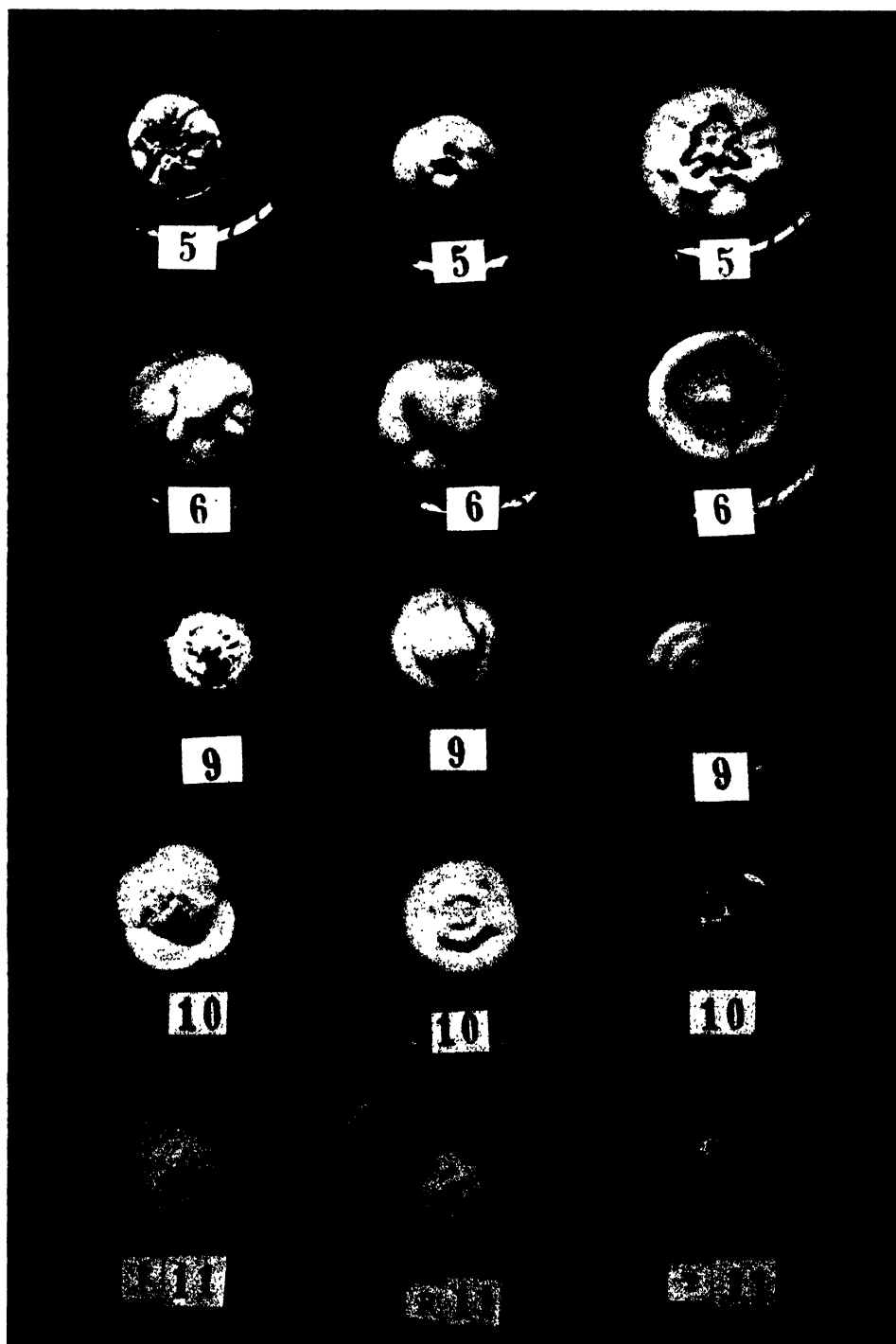
The characteristics of the Kaifeng cultures on 60-day-old potato dextrose agar medium were as follows:—26.1 mm. in average diameter; white to dark olive, or buff in coloration; raised umbonate in topography; coriaceous and

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FIG. 1. The cultural characteristics of 60-day-old colonies of the Kaifeng isolate on various agar media: (1) potato dextrose, (2) Thaxter's hard, (3) 2% maltose potato, (4) 4% sucrose potato, (5) 2% malt extract, (6) 2% malt extract plus 1% dextrose, (7) corn meal dextrose, (8) oatmeal dextrose, (9) carrot decoction, (10) wheat seedling decoction, (11) wheat meal dextrose, and (12) Czapek's synthetic.







*See p. 69 for caption.*



mycelioid in consistency; velvety surface; without zonation; lacerate, lobate, or entire and mycelioid, appressed on margin. The important cultural characteristics of Kaifeng cultures on various media are summarized in Table II and shown in Fig. 1.

A comparative study of cultural characteristics of these cultures (Kaifeng, Hsueh, and Tsetung) on five kinds of artificial media was also made. The results are summarized in Table III. Photographs of 60-day-old colonies are shown in Fig. 2.

The outstanding differences on various media are as follows:—

1. On 2% malt extract agar.

The Kaifeng isolate was usually tulle buff, vinaceous buff to white colored in center, verrucose with furrows leading to the edge in topography, and lobate on the margin. The Hsueh isolate was white and verrucose at the center; other parts of the colony were deep olive buff and smooth. The Tsetung isolate was generally white with tulle buff ridges and a pale olive buff, entire margin.

2. On 2% malt extract plus 1% dextrose agar.

In regard to color, the Kaifeng isolate was pale smoke gray to smoke gray in some portions, the Hsueh isolate was olive buff, and the Tsetung isolate deep olive buff. As to topography, the Kaifeng isolate was umbonate and warty; the Hsueh isolate was pulvinate; and the Tsetung isolate was smooth with convex center. The margin was usually lobate in the Kaifeng isolate, and entire in the others.

3. On carrot decoction agar.

The Kaifeng isolate was pale brownish drab in the central portion and white in other parts, verrucose in topography, and erose on the margin. The Hsueh isolate was white to pale olive buff in color with entire margin, while the Tsetung isolate was characterized by a light buff color, the presence of zones, and the lacerate, raised margin.

4. On wheat seedling decoction agar.

The chief variation of these three isolates on this medium lay in the coloration, which was pale smoke gray in the Kaifeng isolate, white in the Hsueh, and tricolor in the Tsetung.

5. On wheat meal dextrose agar.

The Kaifeng isolate was pallid brownish drab in the central portion, the Hsueh isolate was white in color, and the Tsetung was pale olive buff. As to topography, it was rugose in the Kaifeng isolate, convex in

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FIG. 2. The comparative cultural characteristics of 60-day-old colonies of the Kaifeng (K), Hsueh (H), and Tsetung (T) isolates on various agar media. (5) 2% malt extract, (6) 2% malt extract plus 1% dextrose, (9) carrot decoction, (10) wheat seedling decoction, and (11) wheat meal dextrose.



TABLE II

CULTURAL CHARACTERISTICS OF 60-DAY-OLD COLONIES OF THE KAIFENG ISOLATE ON 12 MEDIA

Media	Characteristics			
	Average diameter* colony in mm.	Coloration	Topography	Margin
Potato dextrose agar	26.1	Umbonate white; other part dark olive to olive buff	Raised; umbonate	Lobate; lacerate, or entire mycelioid; appressed
Thaxter's agar	21.2	Dark olive buff, or citrine drab; white in verrucose part	Raised; verrucose	Slight lacerate; mycelioid; depressed
2% Maltose potato agar	14.1	Brownish drab in central portion, white in the other part	Raised; umbonate; rugose with furrows	Lacerate; mycelioid; appressed
4% Sucrose potato agar	26.4	White to olive buff; verrucose part white	Raised; verrucose	Slight lacerate to lacerate; appressed
2% Malt extract agar	15.6	Tilleul buff; vinaceous buff to white; verrucose part white to pale olive buff	Raised; verrucose to rugose	Lobate; mycelioid; appressed
2% Malt extract plus 1% dextrose agar	23.2	Some part pale smoke gray to smoke gray; other part pale olive buff	Raised; umbonate; warty	Lobate; mycelioid; appressed
Corn meal dextrose agar	17.2	Pale smoke gray to smoke gray	Raised; slight umbonate	Lacerate; mycelioid; papery appressed
Oatmeal dextrose agar	17.5	Pale olive buff; some portions pale smoke gray	Raised; verrucose	Lacerate; mycelioid; appressed
Carrot decoction agar	18.0	Central part pale brownish drab; other part white	Raised; umbonate; verrucose	Erose; mycelioid; appressed
Wheat seedling decoction	19.8	Pale smoke gray	Raised; verrucose	Lobate; mycelioid; appressed
Wheat meal dextrose agar	20.1	Central part pallid brownish drab; other part white	Raised; rugose in center	Entire; mycelioid; appressed
Czapek's synthetic agar medium	20.0	White to pale olive buff	Raised; verrucose to pulvinate at center	Lacerate; mycelioid to bacterioid; appressed

\* Each figure is the average diameter of three separate colonies.

TABLE III  
COMPARATIVE CULTURAL CHARACTERISTICS OF 60-DAY-OLD COLONIES OF THE KAIFENG (K), HSUCHOW (H),  
AND TSETUNG (T) ISOLATES ON FIVE AGAR MEDIA

Media	Characteristics												
	Average diameter* of colony in mm.			Coloration		Topography							Margin
	K.	H.	T.	K.	H.	T.	K.	H.	T.	K.	H.	T.	
2% Malt extract agar (5)	15.6	17.2	25.8	Tilleul buff, vinaceous buff to whitish; verrucose part white to pale olive buff	Center white; other part deep olive buff	White; ridges tilleul buff; margin pale olive buff	Raised; verrucose to rugose	Raised; smooth; verrucose in center	Raised; verrucose with depressed pits at center	Lobate; myceloid; appressed	Slight lacerate; myceloid; appressed	Entire; myceloid; appressed	
2% Malt extract plus 1% dextrose agar (6)	23.2	21.3	22.5	Some portions pale smoke gray to smoke gray; other part white to pale olive buff	Olive buff to deep olive buff	Olive buff	Raised; umbonate; warty	Raised; pulvinate	Raised; smooth; center convex	Lobate; myceloid; appressed	Entire or lacerate; myceloid; raised	Entire myceloid; raised	
Carrot decoction agar (9)	18.0	23.7	24.0	Central part pale brownish drab; other part white	White to pale olive buff	Light buff	Raised; umbonate, verrucose	Raised; umbonate	Raised; umbonate	Erose; myceloid; appressed	Entire; myceloid; appressed	Lacerate; myceloid; raised	
Wheat seedling decoction agar (10)	19.8	23.6	18.2	Pale smoke gray	White	Verrucose wood brown; some portions white; deep olive buff periphery	Raised; verrucose	Center raised; slight verrucose or rugose	Raised; verrucose	Lobate; myceloid; appressed	Lacerate; myceloid; appressed	Lacerate; myceloid; raised	
Wheat meal dextrose agar (11)	20.1	26.5	16.0	Central part pallid brownish drab; other part white	White	Pale olive buff	Raised; rugose in the central part	Raised; center convex	Raised; center umbonate; warty	Entire; myceloid; appressed	Erose; myceloid; appressed	Entire; myceloid; raised	

\* Each figure is the average diameter of three separate colonies.

the Hsuchow, and umbonate and warty in the Tsetung. The margin was usually entire in the Kaifeng isolate, erose in the Hsuchow, and entire and raised in the Tsetung. In addition, the Kaifeng isolate had concentric zonation.

### Conclusions

The foregoing results showed that *U. tritici*, like other smut fungi, could be cultured on various media, although its growth rate was rather slow. The three geographical isolates differed characteristically on five different media.

### Acknowledgments

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## THE CHEMICAL COMPOSITION OF MARINE ALGAE<sup>1</sup>

BY MARGARET G. MACPHERSON AND E. GORDON YOUNG

### Abstract

Fourteen species of the commoner marine algae of Nova Scotia have been analyzed for their content of water, nitrogen, ash, calcium, phosphorus, and iron. These algae are remarkable for their consistently high ash content. The minimum value was 12.3% in *Ahnfeltia plicata* and the maximum 45.4% in *Enteromorpha intestinalis*. The average content was 24.9%. Differences in composition have been observed between the green, red, and brown classes of algae.

It has been estimated that the plants of the sea equal in quantity the plant growth on land. Be that as it may, they have been used by man scarcely at all and may be regarded as a valuable potential natural resource. Our knowledge of the chemical composition of seaweeds, however, is very fragmentary. For Canadian waters of the Atlantic coast there are only the analyses of Butler (3, 4), mainly for six of the commonest species and with particular reference to potassium and iodine.

Within the past 10 years the commercial importance of certain species has become apparent,—notably *Chondrus crispus* (Irish moss) for the production of carrageenin and species of the *Laminariales* (kelp) and *Fucales* (rockweed) for the extraction of alginates. Our ignorance of the general composition of marine algae stimulated this initial survey. It has been directed towards those constituents of nutritional importance because of the increased use of algal extracts in the food industry. Only the commoner species to be found on the coast of Nova Scotia have been analyzed and the selection was based on the survey of Bell and MacFarlane (1).

### Experimental

#### *Preparation of Material*

The plants were transported in sea water from the source to the laboratory. Identification of species was based on the manual of Taylor (16). Each analysis in this study represents a mixed sample prepared from a number of plants collected from the same place at the same time.

The determination of moisture was done on plants from which the adhering water was removed with cheesecloth. Large plants were minced in a food chopper. The error introduced by this procedure is apparently small (3). The plants were dried for 30 to 48 hr. in an oven at 100° C. They were then ground in a Wiley mill to pass through a sieve of No. 20 mesh. Prior to final weighing they were again dried in the oven and placed in a desiccator.

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### Methods

Moisture determination was done on 10 to 40 gm. of the fresh seaweed by drying to constant weight at 100° C.

Total lipids were extracted from dried samples with ether for 16 hr. in a Soxhlet apparatus. The amount found was so small that only six species were analyzed.

The Kjeldahl-Gunning method was used for the determination of total nitrogen on moisture-free samples.

The ash content was found by igniting 2 to 4 gm. in an electric muffle furnace maintained at about 600° C. This required about eight hours.

Phosphorus was determined on approximately 1 gm. of the dried sample after digestion with 60% perchloric acid in a 50 ml. Kjeldahl flask according to the procedure of King (10). When digestion was complete the remaining liquid was made up to 100 ml. in a volumetric flask and a suitable aliquot (containing 0.2 to 0.8 mgm. of phosphorus) transferred to a 100 ml. volumetric flask. The color was developed according to the method of Fiske and Subbarow (6).

For the estimation of calcium, 15 ml. of the mixture after digestion with perchloric acid, referred to in the phosphorus determination, was pipetted into a 25 ml. volumetric flask. This was neutralized with 4 *N* ammonium hydroxide to about pH 6 as shown by bromcresol green. The calcium was then determined by the Clark-Collip modification (5) of the Kramer-Tisdall procedure.

Iron was estimated likewise on the perchloric acid digest by the method of Swank and Mellon (15) with sodium thioglycolate. Under these conditions the aliquot used did not require to be neutralized prior to the development of color.

The difference between the sum of the figures for protein ( $N \times 6.25$ ), lipid, and ash and the total solids is usually assumed to be carbohydrate. Such values are only approximate because it is probably incorrect to calculate protein from the determinations of total nitrogen because of the presence of nonprotein nitrogen and the uncertainty of the level of nitrogen in the protein present (9, 13).

### Results

The complete analyses are presented in Table I together with the site and time of collection. These plants are remarkable for the low level of total lipids that they contain, averaging about 2% in the species analyzed, and for the uniformly high level of ash content and total carbohydrate.

The content of moisture varied between 67 and 90% for all species examined. In two species it was not possible to carry out this determination because of the conditions of collection. The values recorded in Table I are in surprisingly good agreement with previous determinations by Butler (3). Our value for

TABLE I

COMPOSITION OF COMMONER SEAWEEDS OF THE MARITIME PROVINCES EXPRESSED AS PERCENTAGE ON A MOISTURE-FREE BASIS

Species	Moisture	N	Protein (N $\times$ 6.25)	Lipid	Ash	Calcium	Phosphorus	Iron	Class
<i>Alaria esculenta</i> (a)	82.4	2.13	13.3	—	29.2	1.45	0.43	0.026	Brown
<i>Ahnfeltia plicata</i> (b)	—	2.30	14.4	—	12.3	0.83	0.21	0.051	Red
<i>Ascophyllum nodosum</i> (c)	67.3	1.76	11.0	2.47	19.8	1.25	0.16	0.015	Brown
<i>Chondrus crispus</i> (d)	77.0	1.94	12.1	—	26.8	0.91	0.11	0.030	Red
<i>Chordaria flagelliformis</i> (e)	88.9	2.41	15.1	—	27.2	1.53	0.26	0.094	Brown
<i>Dictyosiphon foeniculaceus</i> (f)	88.2	2.56	16.0	—	23.1	2.02	0.24	0.053	Brown
<i>Enteromorpha intestinalis</i> (g)	—	2.58	16.1	—	45.4	0.68	0.21	0.582	Green
<i>Fucus evanescens</i> (c)	86.5	2.70	16.9	1.80	17.7	0.97	0.25	0.055	Brown
<i>Fucus serratus</i> (h)	74.9	0.59	3.7	—	14.5	1.19	0.13	0.044	Brown
<i>Fucus vesiculosus</i> (i)	76.6	1.73	10.8	0.43	21.5	2.93	0.18	0.067	Brown
<i>Gigartina mamillosa</i> (a)	76.2	3.62	22.6	—	21.2	0.51	0.29	0.036	Red
<i>Halosaccion ramentaceum</i> (j)	83.3	3.03	18.9	2.82	19.7	1.04	0.30	0.038	Red
<i>Laminaria Agardhii</i> (j)	88.8	2.09	13.1	1.96	27.2	1.09	0.22	0.015	Brown
<i>Laminaria digitata</i> (a)	86.5	1.69	10.6	—	27.9	1.29	0.23	0.018	Brown
<i>Laminaria longicuris</i> (f)	90.5	1.84	11.5	—	34.8	1.46	0.16	0.030	Brown
<i>Rhodomenia palmata</i> (c)	88.1	4.04	25.3	3.78	26.7	0.24	0.32	0.025	Red
<i>Ulva Lactuca</i> (a)	84.5	3.75	23.4	—	29.1	0.82	0.29	0.139	Green
Averages									
Phaeophyceae (10)	83.0	1.95	12.0	—	24.3	1.52	0.23	0.042	Brown
Rhodophyceae (5)	81.0	2.99	19.0	—	21.3	0.71	0.25	0.036	Red
Chlorophyceae (2)	—	3.17	20.0	—	37.3	0.75	0.25	0.360	Green
General average	83.0	2.40	15.0	2.21	24.9	1.19	0.24	0.078	

## Site and time of collection

- (a) Sandy Cove, N.S., August, 1947.  
 (b) Near West Pubnico, Yarmouth Co., N.S., August, 1948.  
 (c) Halifax, N.S., May, 1947.  
 (d) Offshore near Wedgeport, Digby Co., N.S., August, 1947.  
 (e) Bliss Island, near St. Andrews, N.B., July, 1947.  
 (f) St. Andrews, N.B., August, 1947.  
 (g) West Pubnico, Yarmouth Co., N.S., July, 1948.  
 (h) Piclou, N.S., August, 1947.  
 (i) Halifax, N.S., October, 1946.  
 (j) Halifax, N.S., June, 1947.

*Laminaria longicuris* is 91% as compared to 89; *Rhodomenia palmata*, 88 vs. 85; *Fucus vesiculosus*, 77 vs. 76; *Ascophyllum nodosum*, 67 vs. 70; *Gigartina mamillosa*, 76 vs. 68.

The variation in total lipids was 0.43 to 3.78% in six species and this can be compared with 0.04 to 1.73% as recorded by Reed (14) on air-dried material of Hawaii and 0.5 to 3% in the analyses of four species by Lunde (12). Haas and Hill (7) have observed an increase of total lipid with degree of emergence in different ecological zones.

The values for total nitrogen are close to those obtained by Butler (3). In most cases they are near the mode as listed by Lunde (12) except in the case of *Fucus serratus* where his values are 1.44 to 2.88% as compared with

0.59%. Values for six species are recorded for the first time. The mean for brown seaweeds is appreciably lower than those for red or green.

The variation in total ash content is 12.3 to 45.4% with an average of 24.9%. The highest value obtained was in the green alga, *Enteromorpha intestinalis*, and this suggests that there may be an unusual polysaccharide present in this species. The results also suggest a possible difference in this respect between the green marine algae and those of other classes. The agreement in various analyses for the same species in different parts of the world, collected at different times, is astonishingly good. This is shown in Table II for two species.

TABLE II  
ASH VALUES AS PERCENTAGE OF DRY WEIGHT

	<i>Ascophyllum nodosum</i>	<i>Fucus vesiculosus</i>
Hendrick (8)	21.5	20.3
Butler (3)	22.2	20.7
Lunde (12)	21.0	22.0
Authors	19.8	21.5

The amount of calcium present in algae is appreciable and the analyses suggest a difference between the *Phaeophyceae* at 1.52% and the two other groups at 0.7%. Phosphorus is remarkably constant in all three classes at approximately 0.24%. Calculated from the mean values, calcium would be 4.8% of the ash content and phosphorus about 1%. There are very few such analyses in the literature. Lunde (12) found calcium at 2.95 and phosphorus at 0.18 as % of total solids in *F. vesiculosus* and calcium, 1.24 and phosphorus, 0.16% in *A. nodosum*. In the ash of the fronds of *Laminaria*, calcium constituted 3.5 to 6% and phosphorus, 2 to 4%. These figures are of the same order of magnitude as our own.

The content of iron appears to be much higher in green algae than in the other groups studied. The values obtained by Lunde (12) for *A. nodosum* and *F. vesiculosus* are identical with those in Table I. These appear to be the only analyses in the literature.

These analyses were performed on plants collected for the most part during the summer months. The work of Butler (4) on *Chondrus crispus*, of Lunde (11), and the recent extensive analyses of Black (2) on the *Laminariaceae* suggest important variations dependent on season and habitat. It is hoped to extend these analyses to establish our knowledge of these factors in relation to the species studied and to examine such constituents as proteins and carbohydrates in greater detail.

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## A STUDY OF TROUT STREAMSIDE COVER IN LOGGED-OVER AND UNDISTURBED VIRGIN SPRUCE WOODS<sup>1</sup>

BY R. G. H. CORMACK<sup>2</sup>

### Abstract

In the present study of trout streams in mountainous headwater regions, the term "streamside cover" includes a wide strip of forest vegetation on both sides of the stream, rather than a narrow fringe of plants at the water's edge. Undisturbed stream margins in virgin spruce woods are compared with those in adjacent areas recently logged over in relation to shade production, water conservation, and protection against stream-bank erosion. Heavy, indiscriminate cutting along the stream margins has removed almost all the good shade producing trees, eradicated by exposure and mechanical means the former water conserving ground vegetation, and caused serious damage to the stream itself by logging operations. As the result of this study, the prohibition of all cutting along wide strips on both sides of the trout stream is strongly recommended in order to maintain suitable environmental conditions for trout and to preserve aesthetic values of fishing areas.

### Introduction

A botanical survey, sponsored by the Alberta Department of Lands and Mines, was undertaken during the summer of 1944. The purpose of the survey was to obtain information concerning the vegetation of undisturbed and disturbed forest areas, to analyze the information and to relate it to the problems of soil erosion, water conservation, and trout stream management.

The region involved is a wide tract of mountainous country, designated as the Crowsnest Forest Reserve in southwest Alberta. On the Alberta side of the watershed all the valleys support dense stands of coniferous forest, either virgin or comparable well developed stands of second growth. The forest is of great importance as a protection for this part of the watershed in that it contains the headwaters of many important rivers. For a long time this well watered, forested section of the watershed has been known as one of the most important trout fishing regions in western Canada. However, within recent years there has been a reported decline in the trout populations. This observation, together with reports of the drying up of springs and of periodic floods and drought in the larger streams, indicates a common cause; the disturbance of the forest cover.

Foresters are well aware of the benefits derived from the study of virgin stands. Lutz (18) believes that a virgin stand offers the best place to investigate natural processes as they relate to tree growth and advises foresters to go to the virgin stand for knowledge of forest ecology on which to base their silvicultural practice. Hough (13) is also of the same opinion and states that a study of the climax virgin forest will show the tree species and kind of forest organism capable of using the site to best advantage.

<sup>1</sup> Manuscript received February 2, 1949.

Contribution from the Department of Botany, University of Alberta, Edmonton, Alta.

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In regard to the present survey it was thought that a study of trout stream conditions in a virgin forest would be valuable not only in obtaining a fuller understanding of the changes produced by the disturbance of the forest cover but also in formulating a wise policy of trout stream protection and management. In a study of this kind not only is it essential to know the identity, abundance, and distribution of streamside plants but enquiry should also be made into their capacity to give shade and protection, to conserve soil and moisture, and to harbor food organisms. To attempt to satisfy all the above conditions, unassisted, in a rough terrain, and within a period of two months, was obviously out of the question. Under the circumstances, collecting was mainly restricted to virgin stands along the river valleys. Data in the form of field notes were obtained on the character of the streamside vegetation and forest soil, stream banks, and channels in undisturbed areas and in those areas disturbed by fire, logging, and grazing.

The present paper deals with observations made in only one of the river valleys surveyed, namely that of the Carbondale River. It records the streamside vegetation of undisturbed virgin stands and discusses the changes produced within these stands by logging operations. This paper lays no claim to represent a finished piece of work, but it is hoped that the final result will be a fuller understanding of the influence of the forest cover on the control of stream flow and on the welfare of trout. Trout stream management will be most successful when biologists understand nature's methods and apply them in maintaining a streamside cover best suited to the climate and soil of a given area.

### **Location and Physiography**

The Crowsnest Forest covers an area of approximately 450 square miles in the southwest corner of the Province (Fig. 1). It takes its name from close proximity to the Crowsnest Pass, one of the most famous of Rocky Mountain Passes, and the route followed by the Crowsnest line of the Canadian Pacific Railway. At its widest point the reserve extends eastward from the provincial boundary a distance of about 20 miles and southward from the Crowsnest Pass to a point about 12 miles north of the International boundary.

The greater part of the reserve is very rugged and uneven, since it includes a portion of the Flathead Range of mountains. With the exception of a narrow range of foothills the transition from mountains to plains on the Alberta side of the watershed is very abrupt. The geology of this area according to Hume (15) is mainly of Precambrian strata.

The region is well watered and is drained by the Oldman River, tributary to the South Saskatchewan River system. Traversing the reserve and flowing eastward and north eastward into the Oldman River are the Carbondale River and a westerly branch of the Castle River. However, as mentioned previously this paper deals only with that part of the survey conducted in the Carbondale River valley.

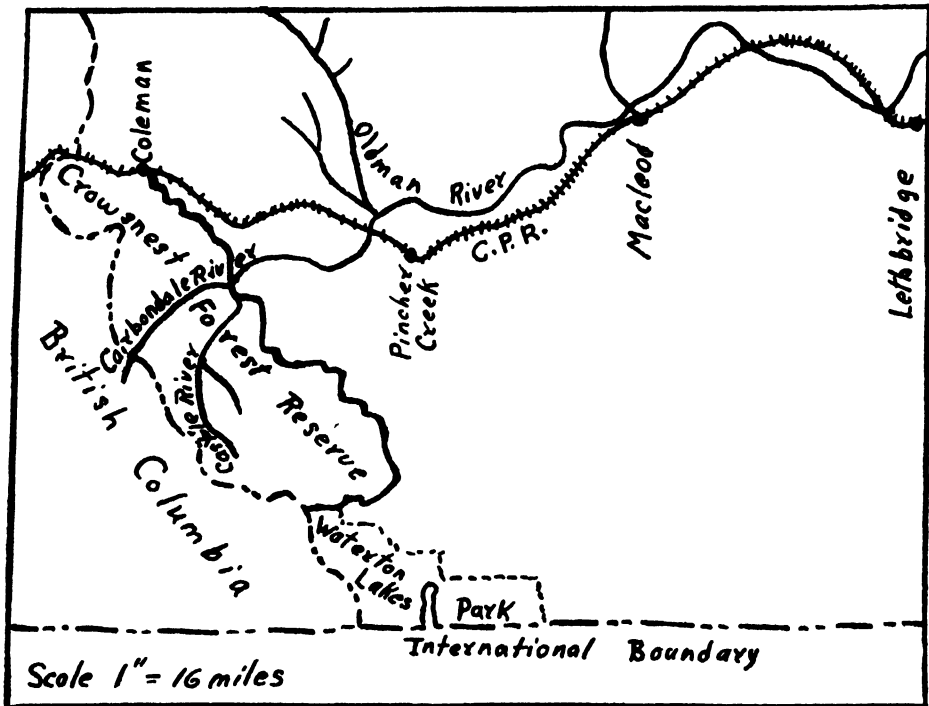


FIG. 1. Map of southwestern Alberta showing location of the Crownsnest Forest Reserve and the Carbondale River.

As shown in Fig. 2 the Carbondale River originates as a small spring-fed mountain stream near the Provincial Boundary and flows eastward through a narrow gap in the mountains designated by Cautley *et al.* (2) as North Kootenay Pass. According to the same authority the "Kootanie Indians" formerly crossed the mountains by this pass each summer to secure a supply of buffalo meat on the Alberta Plains. To-day because of the steepness of the pass particularly from the British Columbia side, this ancient travel route is merely a faint pack-trail much grown over and littered with windfall. On the Alberta side, the pack-trail, which keeps to the north side of the stream, descends open slopes and through dense forest to join a recently constructed road at a point where the latter crosses the Carbondale River. The pass itself is bounded on the north by the southern slopes of Kootenay Mountain (7930 ft.), and on the south by the northern slopes of Mt. McCarty (7748 ft.). Westward, on each side of the pass the mountain slopes are precipitous forming a narrow defile; eastward, the pass opens out and more gentle slopes become apparent. The ridges are separated by wide densely timbered valleys where springs and small tributary streams collect the runoff and flow to MacDonald, Gardiner, and Lost Creeks, which in turn empty into the Carbondale River.

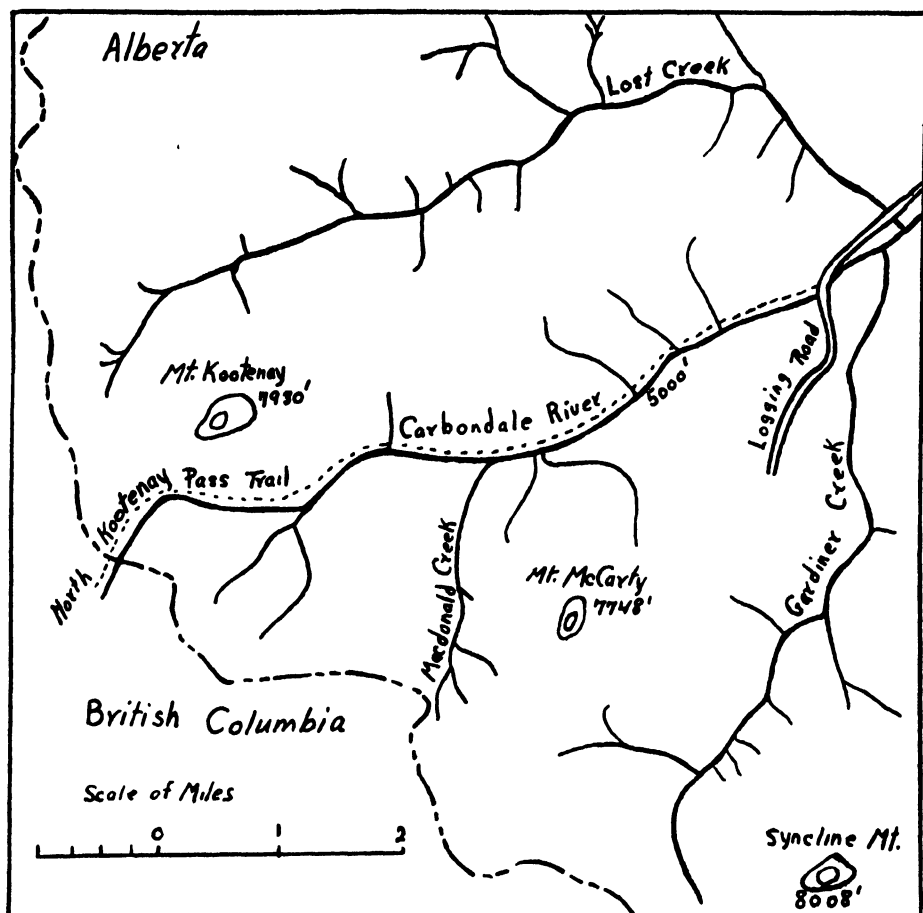


FIG. 2. Map of the study area in the Carbondale River valley.

The area selected for special study included that part of the river valley extending southwest along the river for a distance of about four miles above the mouth of Gardiner Creek, and north and south of here to the valleys of Lost and Gardiner Creeks, respectively (Fig. 2). Although referred to as creeks these two streams are of much the same size as the Carbondale River. Gardiner Creek, which originates at the base of Mt. Syncline (8008 ft.) formed the eastern boundary of the study area. A portion of Gardiner Creek valley was burned in 1936, while Lost Creek Valley is for the most part undisturbed.

Virgin coniferous forest occupies the greater part of the study area and extends west to the British Columbia Border. Spruce is the chief tree on the immediate banks of the river, wide river flats, and the lower level of adjacent slopes. That the species is white spruce (*Picea glauca*) seems most likely, though Engelmann spruce (*Picea engelmanni* (Parry) Engelm.) has been recorded for the same region. Considerable variation in the character of the cones suggests that hybridization occurs between these two species.

The general appearance of such stands is that of a closed, even aged forest characterized by a thick moss cover and by a shrubby and herbaceous undergrowth of variable density and composition. In this regard, these virgin spruce stands closely resemble the northern coniferous forest of eastern Canada described by Nichols (23) as being monotonously uniform over vast stretches of country and impressive in its simplicity. At the eastern end of the study area, aspen poplar (*Populus tremuloides*) occurs as a fringe bordering meadows or with lodgepole pine (*Pinus contorta* var. *latifolia*) in open mixed woods along the south-facing slope of the river. At the western end near the source of the river, lodgepole pine predominates while limber pine (*Pinus flexilis*) occurs on exposed slopes and ridges.

### Undisturbed Streamside Cover

Although the term streamside cover is of common usage no definite statement as to the amount of marginal vegetation involved is to be found in the literature. As the present survey progressed it became evident that a greater portion of the forest vegetation was involved than merely a narrow fringe of plants at the water's edge. Consequently a strip of vegetation approximately 50 ft. in width on each side of the river was arbitrarily decided upon as more truly representing that part of the forest cover effective in providing streamside shade and protection. In typical spruce stands the character of the vegetation within such a narrow strip of ground is strikingly uniform with the exception of minor and irregular differences associated with rocky ledges, springs, and openings in the forest canopy caused by windthrown trees.

A list of plants recorded for undisturbed river margins is presented in Table II. A number of species have been omitted because they were either not mature at the time the survey was made or accurate determinations are not available. However, even if not complete, the list includes most of the characteristic species and certainly those most frequently encountered.

As already mentioned the virgin forest is essentially white spruce, very dense and containing trees of large size and good merchantable value. The largest spruce tree had a D.B.H. of 40 in. while the majority measured from 18 to 30 in., and ranged in height from 100 to 130 ft. In adjacent logged areas the stumps of this species commonly show ages of 200 to 250 years. After spruce, mountain fir is the most common species in the tall tree class. The tallest trees had a height of approximately 90 ft. and had an average D.B.H. of 18 in. Most of the tall fir trees are diseased and such firs constitute the majority of recent windthrown trees on the forest floor. In general seedling reproduction of spruce and fir is poor but reaches its best development under openings in the forest canopy due to the windthrow of overmature trees. Here large numbers of spruce and fir seedlings may be found growing on almost every moss-covered windfall.

Lodgepole pine is frequently present in the streamside coniferous cover but only as a minor constituent and since there is complete lack of reproduction of this species it is clear that lodgepole pine is passing out of the spruce dominated stands. Representatives of this species now in the stands, overshadowed by the spruce, are in a dying condition. That balsam poplar is also disappearing is evidenced by the presence of very few individuals in the tall tree class and none at all in the small tree class. Overtopped trees of this species are common. Douglas fir is of such rare occurrence that it constitutes an unimportant element of the streamside cover. Representative trees are usually taller than the spruce and are in a healthy condition. Two tree species of extremely rare occurrence have been identified as the western cedar and ponderosa pine. These two species are native to British Columbia and have not been listed previously for this part of the Rocky Mountains Forest Reserve (8, 10).

In general, small trees of the leading tree species are few in number and well defined layers or strata of woody plants are poorly developed. Smaller spruce and mountain fir occur as scattered trees of apparently very slow growth in those places where they form part or all of the tall tree canopy. On the immediate banks, often overhanging the water, willows and alders, together with a number of other small trees and shrubs, fill in the intervening spaces between the tall spruce.

The shade provided by this kind of forest cover combined with the usually large amount of soil moisture produces a cool moist atmosphere that is most impressive, and is in decided contrast to adjacent cutover areas. For instance, at midday during the month of July there was an average difference of 33° F. between the atmospheric temperature of an undisturbed virgin stand and one recently denuded of all tall trees. In addition to reducing the rate of evaporation from the forest floor the shade produced by the tall trees is the chief factor determining the floristic composition and density of the ground vegetation.

In undisturbed spruce stream margins, the vegetation on the forest floor is a well developed layer whose composition and character is determined by shade, topography, and proximity to water.

The most characteristic and constant feature of the undisturbed virgin spruce woods is the luxuriant development of mosses, which form a continuous green carpet over the soil, boulders, and windfalls, and at the water's edge over stones, old logs, and projecting tree roots. The luxuriance of this moss carpet some 4 to 6 in. in depth, to those who have never observed it nor walked upon it seems almost unbelievable. The mosses decrease the rate of evaporation from the forest floor; through decay they form more humus and by retarding and absorbing rain and snow they promote the slow seepage of water to the soil beneath. Not only do they soak up water rapidly like a sponge but in the shade of the tall trees they hold it tenaciously even over long periods of dry weather. Along the overhanging banks of the river, springs, and small feeder streams, the mosses check the rapid runoff of surface water, thus preventing erosion and by sifting out silt and debris keep the

water clean and fresh. Typical samples of these mosses were shown on subsequent testing in the laboratory to have a water holding capacity of 300 to 600%. This figure as expressed in percentage represents the amount of water held by 10 gm. of air-dried moss.

The list of mosses (Table I), though by no means complete, includes the most common species and those that constitute the bulk of the moss carpet.

TABLE I

## SPECIES FOUND ALONG UNDISTURBED STREAM MARGINS

(Symbols: A – abundant; C – common; O – occasional; R – rare)

Scientific name	Common name	Abundance
<b>BRYOPHYTES</b>		
	Mosses	
<i>Cratoneuron filicinum</i> (L.) Roth		C
<i>Dicranum Bonjeani</i> De Not.		C
<i>Hylocomium splendens</i> Hedw.		A
<i>Hypnum Crista-castrensis</i> (L.) Hedw.		A
<i>Hypnum Lindbergii</i> Mitt.		A
<i>Hypnum Schreberi</i> Willd.		A
<i>Mnium affine</i> Bland.		C
<i>Mnium punctatum</i> (L.) Hedw.		C
<i>Polytrichum commune</i> Hedw.		O
<i>Polytrichum juniperinum</i> Hedw.		O
<i>Rhytidiadelphus Loreus</i> (L., Hedw.) Warnst.		A
<i>Rhytidiadelphus triquetrus</i> (L., Hedw.) Warnst.		A
<b>PTERIDOPHYTES</b>		
<i>Athyrium Filix-femina</i> (L.) Roth	Lady fern	A
<i>Cystopteris fragilis</i> (L.) Bernh.	Fragile fern	C
<i>Dryopteris disjuncta</i> (Rupr.) Morton	Oak fern	C
<i>Dryopteris spinulosa</i> (L.) Ktze.	Toothed wood fern	C
<i>Pteridium aquilinum</i> var. <i>pubescens</i> (L.) Kuhn.	Bracken fern	R
<i>Woodsia scopulina</i> D.C. Eat.	Mountain cliff fern	O
<i>Equisetum arvense</i> L.	Common horsetail	A
<i>Equisetum hyemale</i> L. var. <i>robustum</i> (A. Br.) A.A. Eat.		
<i>Equisetum pratense</i> Ehrh.	Scouring rush	O
<i>Equisetum scirpoides</i> Michx.	Thicket horsetail	O
<i>Equisetum variegatum</i> Schleich.	Rush horsetail	A
<i>Lycopodium annotinum</i> L.	Variegated horsetail	O
<i>Lycopodium complanatum</i> L.	Bristly club moss	A
	Trailing club moss	C
<b>SPERMATOPHYTES</b>		
(Trees)		
<i>Abies lasiocarpa</i> (Hook.) Nutt.	Mountain fir	C
<i>Acer Douglasii</i> Hook.	Mountain maple	O
<i>Alnus incana</i> (L.) Moench.	Speckled alder	C
<i>Alnus sinuata</i> (Regel.) Rydb.	Alder	C
<i>Picea glauca</i> Voss	White spruce	A
<i>Pinus contorta</i> Loud. var. <i>latifolia</i> S. Wats.	Lodgepole pine	O
<i>Pinus flexilis</i> James	Limber pine	R
<i>Pinus ponderosa</i> Dougl.	Ponderosa pine	R
<i>Populus trichocarpa</i> T. & G.	Western balsam poplar	O
<i>Populus tremuloides</i> Michx.	Aspen poplar	R
<i>Pseudotsuga taxifolia</i> (Poir.) Rehder	Douglas fir	O
<i>Salix glauca</i> L. var. <i>glabrescens</i> (And.) Schn.	Blue-gray willow	C
<i>Salix subcoerulea</i> Piper	Blue-green willow	C
<i>Sorbus scopulina</i> Greene	Mountain ash	O
<i>Thuja plicata</i> D. Don	Western cedar	R

TABLE I—Continued

## SPECIES FOUND ALONG UNDISTURBED STREAM MARGINS—Continued

(Symbols: A – abundant; C – common; O – occasional; R – rare)

Scientific name	Common name	Abundance
<b>SPERMATOPHYTES—Continued</b>		
<b>(Shrubs)</b>		
<i>Amelanchier alnifolia</i> Nutt.	Saskatoon	O
<i>Cornus stolonifera</i> (Michx.) Rydb.	Red osier dogwood	C
<i>Lonicera involucrata</i> (Richards.) Banks	Swamp honeysuckle	C
<i>Menziesia glabella</i> A. Gray	Menziesia	O
<i>Pachystima Myrsinites</i> (Pursh) Raf.	Mountain lover	O
<i>Rhododendron albiflorum</i> (Hook.) Rydb.	Mountain rhododendron	O
<i>Ribes lacustre</i> (Pers.) Poir.	Swamp black currant	O
<i>Rubus idaeus</i> L. var. <i>canadensis</i> Rich.	Wild red raspberry	O
<i>Rubus parviflorus</i> Nutt.	Salmonberry	R
<i>Sambucus melanocarpa</i> A. Gray	Elderberry	O
<i>Shepherdia canadensis</i> (L.) Nutt.	Buffaloberry	O
<i>Spiraea lucida</i> Dougl.	Spiraea	R
<i>Symphoricarpos</i> sp.	Snowberry	R
<i>Vaccinium caespitosum</i> Michx.	Dwarf bilberry	O
<i>Vaccinium membranaceum</i> Dougl.	Bilberry	O
<i>Vaccinium orcopilum</i> Rydb.	Huckleberry	R
<i>Vaccinium scoparium</i> Leiburg	Gooseberry	R
<i>Viburnum eradiatum</i> (Oakes) House	Mooseberry	O
<b>(Sedges, rushes, and grasses)</b>		
<i>Carex atrovirens</i> Mack.	Black-scaled sedge	C
<i>Carex aurea</i> Nutt.	Golden sedge	C
<i>Carex Backii</i> Boott.	Back's sedge	O
<i>Carex Deweyana</i> Schwein.	Dewey's sedge	C
<i>Carex disperma</i> Dewey	Soft-leaved sedge	C
<i>Carex flava</i> L.	Yellow sedge	C
<i>Carex interior</i> Bailey	Inland sedge	C
<i>Carex lenticularis</i> Michx.	Lenticulate sedge	O
<i>Carex Mertensii</i> Prescott	Merton's sedge	C
<i>Carex Rossii</i> Boott.	Ross's sedge	O
<i>Carex rostrata</i> Stokes	Rostrated sedge	C
<i>Juncus balticus</i> var. <i>montanus</i> Engelm.	Baltic rush	C
<i>Juncus longistylis</i> Torr.	Long-styled rush	C
<i>Juncus Mertensianus</i> Bong.	Slender-stemmed rush	O
<i>Juncus saximontanus</i> A. Nels.	Rocky mountain rush	C
<i>Luzula campestris</i> D.C. var. <i>multiflora</i> (Ehrh.) Celak.	Many-flowered wood rush	C
<i>Luzula parviflora</i> (Ehrh.) Desv.	Small-flowered wood rush	C
<i>Agrostis exarata</i> Trin.	Spike reedtop grass	R
<i>Calamagrostis canadensis</i> (Michx.) Beauv.	Marsh reed grass	O
<i>Cinna latifolia</i> (Trev.) Griseb.	Wood reed grass	O
<i>Elymus glaucus</i> Buckley	Smooth wild rye	O
<i>Glyceria</i> (?) <i>elata</i> (Nash) Hitch.	Slender wood grass	O
<i>Glyceria pauciflora</i> Presl.	Manna grass	O
<i>Melica spectabilis</i> Scribn.	Purple onion grass	O
<i>Melica subulata</i> (Griseb.) Scribn.	Melic grass	O
<i>Oryzopsis asperifolia</i> Michx.	Mountain rice	O
<b>(Herbaceous plants)</b>		
<i>Actaea</i> spp.	Baneberry	C
<i>Angelica Dawsoni</i> S. Wats.	Angelica	O
<i>Aquilegia flavescens</i> S. Wats.	Mountain columbine	O
<i>Arnica cordifolia</i> Hook.	Heart-leaved Arnica	C
<i>Aster</i> spp.	Aster	O
<i>Calypso bulbosa</i> (L.) Oakes	Calypso orchid	R
<i>Cardamine pennsylvanica</i> Muhl.	Bitter cress	O
<i>Castilleja mineata</i> Benth.	Indian paint brush	R



TABLE I—*Concluded*SPECIES FOUND ALONG UNDISTURBED STREAM MARGINS—*Concluded*

(Symbols: A – abundant; C – common; O – occasional; R – rare)

Scientific name	Common name	Abundance
<b>SPERMATOPHYTES—<i>concluded</i></b>		
(Herbaceous plants)— <i>concluded</i>		
<i>Chimaphila occidentalis</i> Rydb.	Pipsissewa	R
<i>Clintonia uniflora</i> (Schult.) Kunth.	One-flowered Clintonia	A
<i>Corallorrhiza trifida</i> Chatelain	Early coralroot orchid	R
<i>Cornus canadensis</i> L.	Bunchberry	A
<i>Disporum trachycarpum</i> S. Wats.	Disporum	C
<i>Epilobium angustifolium</i> L.	Fireweed	O
<i>Epilobium</i> spp.	Fireweed	O
<i>Erigeron salsuginosus</i> (Richards.) A. Gray	Fleabane	O
<i>Fragaria glauca</i> (S. Wats.) Rydb.	Wild strawberry	O
<i>Galium triflorum</i> Michx.	Sweet scented bedstraw	O
<i>Geranium Richardsonii</i> Fisch. & Trautv.	White-flowered geranium	R
<i>Geum</i> sp.	Avens	R
<i>Glycosma occidentalis</i> Nutt.	Glycosma	R
<i>Goodyera decipiens</i> Hubbard	Rattlesnake plantain orchid	R
<i>Habenaria dilatata</i> (Pursh.) Hook.	Tall leafy white orchid	C
<i>Habenaria obtusata</i> (Pursh.) Richards.	Blunt-leaf orchid	O
<i>Habenaria saccata</i> Greene	Green bog orchid	C
<i>Heracleum lanatum</i> Michx.	Cow parsnip	O
<i>Heuchera cylindrica</i> var. <i>septentrionalis</i> R.B.L.	Alumroot	R
<i>Lathyrus ochroleucus</i> Hook.	Cream-colored vetchling	O
<i>Linnaea americana</i> Forbes	Twinflower	A
<i>Listera convallarioides</i> (Sw.) Torr.	Twayblade orchid	R
<i>Listera cordata</i> (L.) R. Br.	Twayblade orchid	R
<i>Listera caurina</i> Piper	Twayblade orchid	R
<i>Mimulus guttatus</i> DC.	Yellow monkey flower	R
<i>Mimulus Lewisii</i> Pursh.	Crimson monkey flower	R
<i>Mitella nuda</i> L.	Bishop's cap	C
<i>Mitella pentandra</i> Hook.	Bishop's cap	O
<i>Moneses uniflora</i> (L.) A. Gray	One-flowered wintergreen	O
<i>Osmorrhiza obtusa</i> (Coul. and Rose) Fernald	Sweet cicely	C
<i>Parnassia fimbriata</i> Konig.	Grass of Parnassus	O
<i>Pedicularis bracteosa</i> Benth.	Lousewort	C
<i>Phaca americana</i> (Hook.) Rydb.	Arctic milk vetch	R
<i>Pyrola asarifolia</i> Michx.	Liver-leaf wintergreen	C
<i>Pyrola bracteata</i> Hook.	Purple-flowered wintergreen	R
<i>Pyrola chlorantha</i> Swartz.	Greenish-flowered wintergreen	C
<i>Pyrola minor</i> L.	Small-flowered wintergreen	R
<i>Pyrola secunda</i> L.	One-sided wintergreen	O
<i>Ranunculus Bongardi</i> Greene	Wood buttercup	O
<i>Saxifraga arguta</i> D. Don.	Saxifrage	O
<i>Saxifraga Mertensiana</i> Bong.	Saxifrage	O
<i>Senecio triangularis</i> Hook.	Brook ragwort	C
<i>Smilacina racemosa</i> (L.) Desf.	False spikenard	C
<i>Smilacina stellata</i> (L.) Desf.	Star-flowered Solomon's seal	C
<i>Stenanthium occidentale</i> A. Gray	Stenanthium	C
<i>Streptopus amplexifolius</i> (L.) DC.	Twisted stalk	A
<i>Thalictrum megacarpum</i> Torr.	Meadow rue	C
<i>Tiarella unifoliata</i> Hook.	False miterwort	C
<i>Trollius albiflorus</i> (A. Gray) Rydb.	Globeflower	O
<i>Urtica gracilis</i> Ait.	Slender nettle	O
<i>Veratrum Eschscholtzianum</i> (R. & S.) Rydb.	False hellebore	C
<i>Veronica americana</i> Schwein.	American brooklime	C
<i>Viola glabella</i> Nutt.	Yellow-flowered violet	A
<i>Viola nephrophylla</i> Greene	Northern bog violet	A
<i>Zygadenus elegans</i> Pursh.	White camass	O

Closely associated with the mosses are lichens and liverworts. The chief and most abundant lichen is *Peltigera aphthosa* (L.) Willd., while many others including various species of the so-called reindeer moss (*Cladonia* spp.) are common on moss-covered windfalls and on the trunks of trees.

Windfalls form a constant and characteristic part of the mossy ground cover in all undisturbed river margins. Partly decayed when the trees fall, windfalls decompose further on the ground and within a short time they become covered by a thick mat of mosses, liverworts, and lichens. In general, throughout the stand and particularly on gentle slopes leading down to the river's edge, they aid greatly in checking the runoff of rain and melting snow. Decaying, punky windfall material has a water-holding capacity of 435%. In one instance windfalls of this kind were still wet after a dry period of 20 days during the month of July.

Closely associated with the moss vegetation, from which it is partly derived, is the forest soil. Beneath the mosses as seen in profile is a well defined, black organic layer consisting of partially or wholly decayed vegetable matter.

By virtue of the humus the organic layer possesses a loose, porous texture, which increases permeability and enables it to absorb large quantities of rain and to conduct the water downwards, and into small springs. In undisturbed spruce woods the organic layer varies in thickness from  $1\frac{1}{2}$  to  $2\frac{1}{2}$  in. and has a water-holding capacity of 310%. In sharp contrast to the organic layer, the underlying light colored mineral soil is usually claylike in texture and is frequently securely packed. It has a water-holding capacity of 48% and when the protective moss and organic covering is removed it soon becomes baked hard in the sun and almost impervious to water.

In general the undergrowth of shrubby and herbaceous plants is poorly developed (Table I). As mentioned already there is a close relationship between the density of the undergrowth and the amount of direct sunlight reaching the forest floor. Where the tree canopy is completely closed it is either nonexistent or very thin, but improves as the sunlight increases towards the river's edge, along game trails, and in openings in the tree canopy caused by wind-thrown trees.

On the whole, pronounced grouping or dominance of single species, with the exception of localized communities, is not evident; instead differences in the frequency of plant groups is more apparent. For instance in typical spruce stands the thick moss carpet is characterized by a thin scattering of shrubs, ferns, horsetails, club mosses, and a small but fairly constant number of herbaceous flowering plants. In the vicinity of springs, and in and around wet boggy ground, ferns, horsetails, and sedges are most plentiful, accompanied by orchids and other rare plants. Grasses are absent except along game trails and localized open areas. Near the river margin the mossy overhanging banks are characterized by a growth of shrubs, ferns, horsetails, sedges, and various shade-loving flowering plants. Along the more densely shaded parts of the river these same kinds of plants extend down to the water

where together with a few scattered grasses they constitute a thin interrupted fringe among moss-covered stones and partly submerged windfalls. Where the river widens, particularly at a bend, the shrubby and herbaceous cover reaches its best development and includes a number of flowering plants quite commonly found in open spaces.

Although all the plants listed in Table I are characteristic, the herbaceous species, which together with the mosses, ferns, horsetails, club mosses, and sedges appear to stand out most clearly as indicating the moist cool environment of spruce streamside margins, are: *Streptopus amplexifolius*, *Stenanthium occidentale*, *Clintonia uniflora*, *Cornus canadensis*, *Linnaea americana*, all the orchids and wintergreens. Certainly these plants are the first to wither and perish from lack of shade on the removal of the tall trees.

### THE RIVER

The Carbondale River as it flows through the undisturbed virgin forest is a typical meandering mountain stream. In the study area the river varies in width from 15 to 25 ft. and in midstream from 1 to 2 ft. in depth. With the exception of a few old established beaver ponds the water flows swiftly over a clean stony, gravelly bottom completely free from green scum (green algae) and water weeds (Fig. 3). Under the protective tree cover a luxuriant growth of mosses, ferns, horsetails, and shade-loving flowering plants cover the overhanging river margins and spread downward over the stones and windfalls to the water's edge (Fig. 4). Springs and small feeder streams run crystal clear over clean gravelly beds, in narrow deep-seated channels often covered by mosses, ferns, windfalls, and protecting tree roots. In this way water that seeps slowly into springs and creeks reaches the river with the minimum of loss.

In this stream, shade is always present, though in varying degrees of density. The amount of variation is due to differences in topography, to differences in the grouping and spacing of both trees and shrubs, and again to the presence of gaps in the forest canopy resulting from the windthrow of over-mature trees.

Shade affects trout indirectly by keeping the temperature of the water cool. Available information on trout stream management indicates that temperature is the most important factor determining the distribution of trout (11, 14, 25). The maximum temperature for natural self-sustaining brook trout waters has been set at about 66° F. When the temperature goes higher than this for any considerable period of time the trout disappear. Those streams containing water at the maximum temperature for trout are more favorable than very cold waters where the food supply is limited. Lower temperatures favor spawning but where the temperature of the stream remains low at all times the trout population may become resident and of slow growth. It has also been noted that brook trout are less abundant and usually disappear where they have numerous summer associates (minnows, suckers, etc.). These associates are added in a regular order, the increase in numbers being correlated directly with a rise in the temperature of the water.

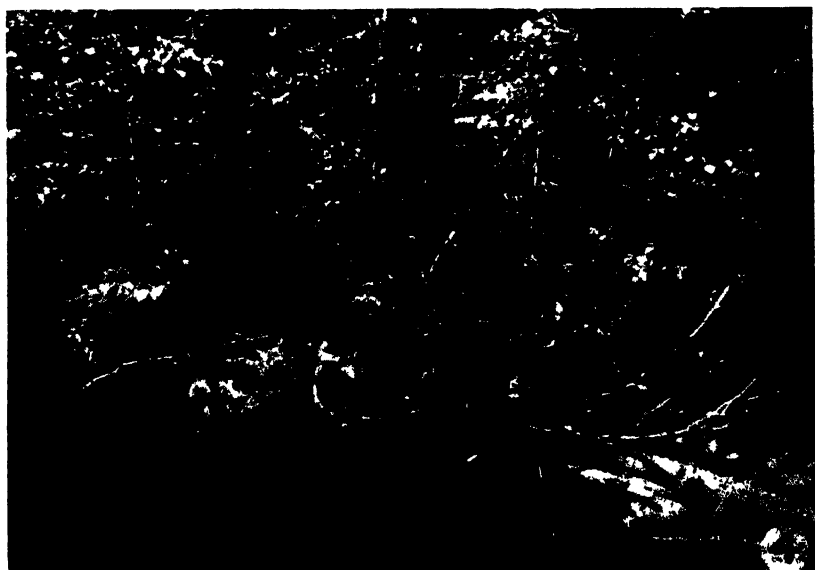
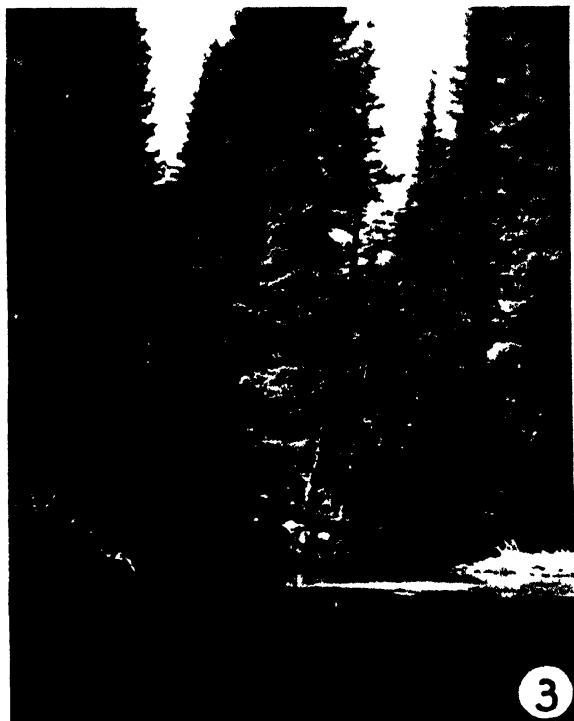


FIG. 3. *The Carbondale River in undisturbed virgin spruce woods.*

FIG. 4. *Shady river margin shown in Fig. 3 with tall spruce on the immediate bank, alders and willows overhanging the river, and a thick continuous carpet of absorbent mosses extending over the ground to the water's edge.*



In the present survey a number of temperature readings shows that the water temperature of the Carbondale River satisfies the conditions necessary for the good growth of trout. During the month of July, the average daily water temperature in undisturbed spruce stands was 47° F. and in beaver ponds, under the same type of cover 53° F. The study did not include adjacent, less densely wooded areas and open beaver meadows, which, until logging operations began in this region, were easily accessible to trout. From the data obtained along the Carbondale River, the height of the streamside cover was found to be an important factor for the production of streamside shade. In the writer's opinion the growth of a few tall spruce near the water's edge is much more effective in this regard than a dense low growth of willows, alders, and shrubs. As the survey progressed it became increasingly clear that the arrangement of tall trees, small trees, and shrubs as they grow in undisturbed virgin forest is the best combination in producing a variable amount of streamside shade and in keeping the temperature of the water below the maximum tolerated by trout. The occurrence of a fair number of Rocky Mountain whitefish and trout in this part of the river strengthened the validity of this opinion.

A study of the relationship of the forest streamside cover to the kind and abundance of trout food did not lie within the scope of the present survey. Although studies in this field are still in the pioneer stage it is known that a considerable amount of trout food, mainly insects, comes from the land (12, 20, 21, 22). In a recent study of Wisconsin trout streams, Anderson (1) concludes that trout will select a given area more from the standpoint of food than of cover, although these two factors may go hand-in-hand.

### **Logged Streamside Cover**

The cutting of a portion of the virgin forest under investigation provided an excellent opportunity to compare and to contrast conditions in both uncut and cut-over virgin stands in the same region.

The cutting of mature timber, mainly white spruce, but also some Douglas fir and lodgepole pine in the Carbondale River valley began in 1943 and was still in progress during the time of the survey. By the beginning of the spring of 1944, the whole width of the river flat extending for a distance of about one-half mile east of Gardiner Creek road, and about one-quarter mile west had been cut, as well as a wide area extending south of the river for nearly a mile (Fig. 2). During early spring of 1944 logging operations were continued on both sides of the road and by the month of July had extended both south and southwest toward the area of Macdonald Creek.

It is well known that the cutting of mature trees alters the natural forest conditions and that no matter how carefully the work is done there is always a certain amount of damage to the remaining vegetation. According to Dwight (8) ordinary logging in spruce stands nearly always opens up the stand too much, leaving only scattered seed trees, with the result that the original

moss cover dries out and the growth of grass and other unfavorable plants is encouraged. Heavy logging as exemplified in the Carbondale River valley brings about still more marked changes along the lines indicated above for ordinary logging. Here, because timber berths sold under old Dominion licenses allowed for clear cutting to a 7-in. diameter, all the good spruce and Douglas fir trees have been cut, leaving uncut the injured, deformed, poor grade trees, and species of low merchantable value, mainly fir and pine. However, if not typical for the whole reserve, the cutting of timber in this area is a well defined example of the maximum amount of damage that can be caused to the streamside cover of a first-class trout stream by heavy, indiscriminate cutting.

The most obvious change is the drying out and subsequent death and disappearance of the mosses, ferns, horsetails, club mosses, and shade-loving flowering plants. In some areas the mosses and the layer of black surface soil, in drying out, have separated from the yellow claylike subsoil and although the surface layers still soak up water during rain they lose it soon afterwards by evaporation. In other areas the surface layers have been mechanically stripped from the subsoil by the action of bulldozers, while in still others they have been obliterated under piles of debris, slashings, and uprooted trees. This accumulation of rubbish, together with piles of sawdust, constitute a serious threat of future fires. With the removal of the mossy ground cover and black surface soil the exposed subsoil becomes baked hard in the sun, and water, which formerly seeped slowly underground, now either runs off rapidly or collects in stagnant pools and evaporates.

The changes produced along the margins of the Carbondale River can best be described with reference to Figs. 5, 6, 7, and 8. Throughout the logged-over area every sound tree has been cut at the water's edge. Those that remain are poor grade trees and many of these with their roots damaged have since blown down or are in a dying condition. Replacing the former community of mosses and associated shade-loving plants, are species formerly less abundant or completely excluded (Table II). Plants in the first category that appear to thrive better under conditions of increased sunlight include some of the small trees and shrubs and a number of the hardier herbaceous species. New plants that are progressively coming in either from adjacent aspen woods and meadows or being introduced as a result of logging operations are mainly weeds and coarse grasses. Thus formerly forested streamside are gradually changing to a wilderness of willows and weeds. This new type of streamside cover produces scarcely any shade and because it is much less effective in retarding the runoff of surface water, streambank erosion is well advanced. In this area the construction of logging roads has accelerated soil erosion, causing heavy silting of the river channel and of the water itself. Logging roads and sunken causeways add to the destruction of the former mossy vegetation and greatly disturb the flow of springs and small feeder streams. Consequently much water no longer reaches the river but settles in shallow muddy pools and evaporates. Chamberlain (3) and Tarzwell as

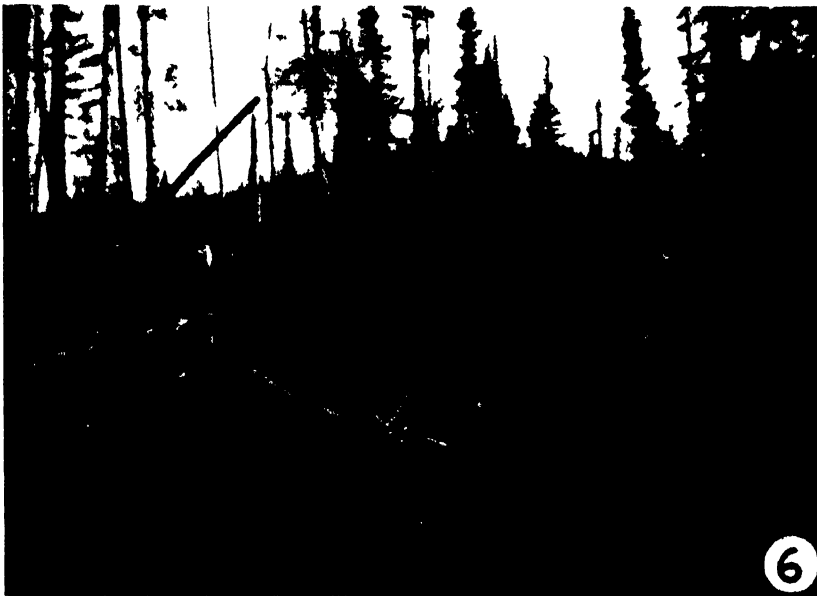
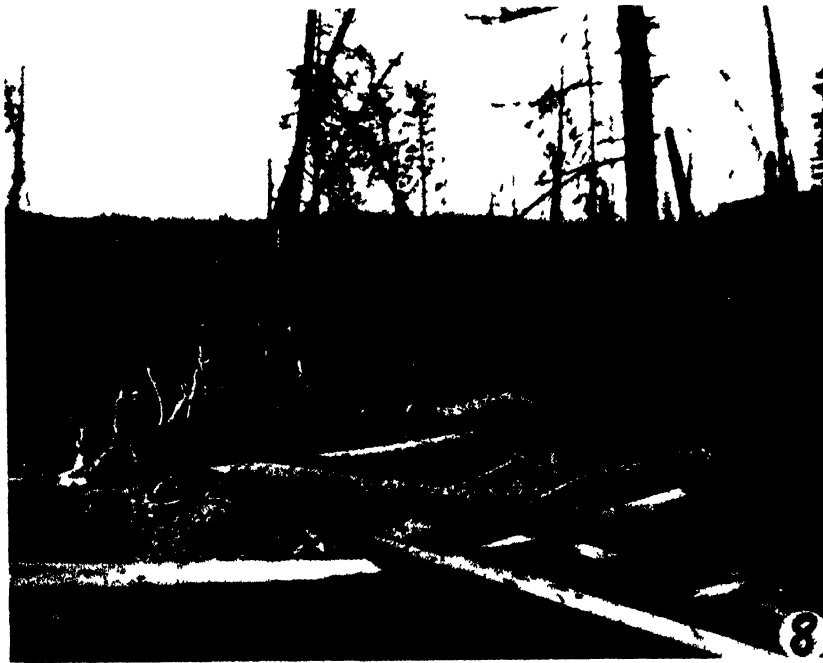


FIG. 5 *The Carbondale River in a logged-over area.*

FIG. 6 *Exposed muddy river margin shown in Fig. 5, littered with debris and with the former water-conserving ground vegetation completely destroyed. Stream-bank erosion is well advanced.*





FIGS 7 and 8 The Carbondale River in logged-over area, showing removal of all sound shade-producing trees and the river channel blocked completely over long distances.

TABLE II

SPECIES FOUND ALONG LOGGED-OVER STREAM MARGINS

Scientific name	Common name	Abundance
<i>Agropyron repens</i> (L.) Beauv.	Quackgrass	C*
<i>Alnus incana</i> (L.) Moench.	Speckled alder	C
<i>Alnus sinuata</i> (Regel.) Rydb.	Alder	C
<i>Amaranthus</i> sp.	Pigweed	O*
<i>Amelanchier alnifolia</i> Nutt.	Saskatoon	O
<i>Arabis glabra</i> (L.) Bernh.	Rock cress	O*
<i>Arnica cordifolia</i> Hook.	Arnica, heart-leaved	C
<i>Aster</i> spp.	Asters	A
<i>Beckmannia syzigachne</i> (Steud.) Fern.	Slough grass	O*
<i>Bromus pumpellianus</i> Scribn. etc.	Brome grass	A*
<i>Calamagrostis canadensis</i> (Michx.) Beauv.	Marsh reed grass	C
<i>Calamagrostis inexpansa</i> A. Gray	Northern reed grass	O*
<i>Calamagrostis rubescens</i> Buckl.	Pine grass	O*
<i>Chenopodium album</i> L.	Lambs'-quarters	C*
<i>Cirsium arvense</i> (L.) Scop. etc.	Canada thistle	C*
<i>Cirsium</i> spp.	Thistles	C*
<i>Cornus stolonifera</i> (Michx.) Rydb.	Red osier dogwood	A
<i>Elymus glaucus</i> Buckley	Smooth wild rye	C
<i>Epilobium angustifolium</i> L.	Fireweed	A
<i>Erigeron</i> spp.	Fleabanes	C*
<i>Erysimum cheiranthoides</i> L.	Wormseed mustard	C*
<i>Fragaria glauca</i> (S. Wats.) Rydb.	Wild strawberry	O
<i>Geranium Bicknellii</i> Britt.	Wild geranium	O*
<i>Gilia gracilis</i> Hook.	Gilia	O*
<i>Heracleum lanatum</i> Mich.	Cow parsnip	C
<i>Osmorrhiza obtusa</i> (Coult. & Rose) Fernald	Sweet cicely	C
<i>Phleum pratense</i> L.	Timothy	C*
<i>Polygonum</i> spp.	Knotweeds	C*
<i>Populus</i> spp.	Poplars	O
<i>Prunella vulgaris</i> L.	Selfheal	O*
<i>Ribes lacustre</i> (Pers.) Poir.	Swamp black currant	O
<i>Rosa</i> spp.	Wild roses	O*
<i>Rubus idaeus</i> L. var. <i>canadensis</i> Rich.	Wild red raspberry	C
<i>Rubus parviflorus</i> Nutt.	Salmonberry	C
<i>Salix glauca</i> L. var. <i>glabrescens</i> (And.) Schn.	Blue-gray willow	C
<i>Salix subcoerulea</i> Piper	Blue-green willow	C
<i>Senecio pseudolaureus</i> Rydb.	Thin-leaved ragwort	C*
<i>Senecio triangularis</i> Hook.	Brook ragwort	C
<i>Silene Menziesii</i> Hook.	Catchfly	O*
<i>Spiraea lucida</i> Dougl.	White meadowsweet	C
<i>Symphoricarpos</i> sp.	Snowberry	C
<i>Taraxacum officinale</i> Weber	Dandelion	C*
<i>Urtica</i> spp.	Nettles	C

\* Introduced species.

quoted by Davis (7) contend that the heavy silting of the water as the result of streambank and roadbank erosion decreases the trout-carrying capacity of all streams and may render the planting of new trout in some streams ineffective.

In this region no attempt has been made to safeguard the river. Slash, whole trees, and debris of all kinds have been piled or thrown into the water (Figs. 7 and 8). In many instances this practice has resulted in the clogging of the river for long distances and there is more than one example where the

river has been so thoroughly blocked that the water has been forced into a new channel. Indeed if it were done deliberately the destruction of the Carbondale River in the cut-over area could not be more complete.

Whether seedlings of spruce, fir, and lodgepole pine will be able to germinate and grow in the cut-over areas is a question that could not be settled at the time the survey was made. However, the widespread destruction of the surface soil, the almost complete absence of sound seed trees, and high soil temperatures indicate that natural reproduction of coniferous trees has been put back for a great many years.

### **Conservation Problems**

Remote mountain and foothill streams have long been associated with good trout fishing in the minds of anglers. However, to-day, in many such streams, decreased stream volume and unstabilized flow of water are causing heavy losses of trout. With the realization that these deleterious changes in water supply are related to changes in the vegetative cover (5, 6, 9, 17, 24, 25, 27, 28) more attention is being given by experts in stream management to the study of conditions in the headwater regions. For instance, Tarzwell, as quoted by Davis (7), believes that stream improvement means environmental improvement. In order to have lasting stream improvement, in his opinion, it is necessary to get out on the watershed and remedy as far as possible the unfavorable conditions that have damaged the streams. Feast, as quoted by the same authority (7), is convinced from his experience in managing Colorado trout streams that any stream that has a good watershed is a good trout stream and any so-called improvement work upon it would prove to be a needless expense. Miller (19), in a recent article summarizing his observations of Canadian trout streams, states: "Effective watershed protection, restoration of vegetative cover along denuded headwaters and other stream improvement measures give more promise of better fishing than rearing fish in hatcheries and dumping them into unsuitable waters."

Evidence obtained in the present survey of the Carbondale River shows that the streamside cover in undisturbed virgin spruce stands has a twofold influence, first, as it influences the conservation of water as an integral part of the watershed protection forest, and second, as it influences the stream itself.

The advantages of this type of forest cover in controlling the flow of water, and the detrimental changes produced in this regard as the result of logging operations have been discussed briefly above. Although the importance of implementing sound forestry practices in the cutting of timber in valuable watershed areas cannot be overemphasized, a thorough discussion of these practices does not lie within the scope of the present paper. Suffice it to say here, that with the accumulation of knowledge on restoration and improvement of watersheds, foresters are placing more emphasis on the relation of forestry to water supplies. In this connection, Connaughton and Wilm (4) describe certain timber-cutting methods that achieve the double purpose of harvesting

a good crop of timber while enhancing stream flow. They point out however, that the practice of forest cutting to improve water supplies cannot be applied indiscriminately. "If floods and erosion offer no particular problem and regulation of flow is achieved by reservoirs, the cut should be frequent and as heavy as may be compatible with acceptable economic returns. If natural regulation of flow is more important than water-yield, cutting should be lighter and a fairly complete forest canopy should be maintained. If flood and erosion hazards are high, cutting must be held to a minimum—no more than enough to keep the forest healthy and productive." With regard to headwater streams such as the Carbondale River it would appear that natural stream flow is most desirable to maintain satisfactory stream conditions for trout. Consequently cutting should be conservative, intelligently managed, and closely supervised.

The particular advantages of undisturbed virgin stands in providing shade and protection to the stream itself have been dealt with above. However, the value of shade in promoting the luxuriant growth of absorbent mosses, and regulating water temperatures for trout is worthy of special consideration. For the production of streamside shade it is height of the vegetative cover that counts and the forest cover bordering the Carbondale River has this characteristic by virtue of the giant spruce. In certain places, particularly where the river is comparatively narrow, shade may be almost continuous. Dense, unbroken shade over the whole length of a trout stream according to Huntsman (16) may not be desirable from the standpoint of trout food, but in mountainous headwater regions this condition seldom arises. Taking the Carbondale River as an example, topographic differences and modification of the forest cover by fire and by the grazing of game animals since time immemorial all go to produce variable environmental conditions for trout with different amounts of shade. Before cutting began in this part of the Carbondale River valley, deep shaded pools, less densely shaded shallow riffles and eddys, stretches of open comparatively quiet water, and numerous tributaries were accessible to trout. Now, as the result of logging operations, streambank and road bank erosion has caused heavy silting of the former gravelly channel over long distances, while the clogging of the river at numerous points, and the loss of the protective cover has seriously interfered with the feeding and spawning habits of trout by greatly restricting their movements. Apart from the standpoint of shade and protection the value of the virgin forest streamside cover in maintaining the natural beauty and attractiveness of the river cannot be overemphasized and in this respect alone is greatly superior to the rank low growth of weeds, willows, and shrubs that invariably follows logging operations.

The cutting of timber is by no means the only cause of disturbance to the streamside cover in watershed areas, but since it comes directly under the jurisdiction of the watershed manager, suitable protective measures can be

devised and barring fire can be rigidly enforced. From evidence obtained in the present survey one measure of stream protection that seems most desirable would be the prohibition of all cutting along wide strips on both sides of the stream. There is considerable precedent for advising a policy of this kind, as multiple use forestry admits noncutting in certain areas, if it is genuinely needed (17, 26). The width of the strips to be left uncut will undoubtedly vary with the individual stream and with the type of forest cover. Taking conditions in the Carbondale River Valley as more or less general for this part of the watershed the writer suggests as a beginning point a strip of at least 60 ft. on each side of the stream. Certainly the uncut areas should be wide enough to provide the maximum of shade and protection to both stream and streamside cover and to preserve the natural attractiveness of the stream. Also they should be extensive enough to include the stream's source, springs, and small feeder tributaries. This protective strip will also provide a much needed supply of tree seeds. Consideration for the protection of the stream must be made well in advance of logging operations and whatever the optimum width of the protective border may be, the watershed manager will be obliged to include its attainment in his plans.

Confirmatory evidence that protective strips should be of a good width was obtained by a study of streamside conditions in the adjoining valley of the Castle River. In 1936 a fire destroyed the whole valley with the exception of strips of unburned trees bordering both sides of the river. Where the strips are extremely narrow some shade is still provided by the tall trees but because of the poor condition of the ground cover, streambank erosion has started and springs and small feeder streams have dried up. Where the unburned strips measure 60 to 100 ft. or more in width, conditions bordering the river are identical to those in undisturbed virgin spruce woods.

Although the stream must be protected against mechanical damage, overall stream protection can only be achieved by the wise management of the whole watershed. In short, if the water-holding capacity of more or less undisturbed forest areas are maintained at a high level and that of burned and cutover areas restored, there will be no cause for concern about the streams themselves nor about the welfare of trout.

### Acknowledgments

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## ELECTROPHORETIC PROPERTIES OF PEA PROTEINS<sup>1</sup>

BY L. R. WETTER<sup>2</sup> AND A. G. MCCALLA<sup>3</sup>

### Abstract

The soluble proteins of pea meal were extracted with phosphate buffer and fractionally precipitated with ammonium sulphate. An electrophoretic analysis of each preparation was made, and the amount and mobility of each component determined. There are probably three distinct components in the pea protein and one of these at least is a protein system, which tends to separate into further components under some conditions. The components were not at all well separated by fractional precipitation, and the separation was decidedly affected by the method of preparation and the pH of the precipitation medium.

### Introduction

Very little work on the nature of the proteins in peas has been carried on since Osborne and Harris (5) published the results of an investigation on fractionation. These and earlier (4) results indicated that there were three distinct proteins in peas, two of them, legumin and vicilin, being globulins, and the third, legumelin, being an albuminlike protein. As with most of Osborne's work, the criterion of distinction used was solubility of the fractions in salt solutions, and it was believed that the proteins prepared using fractional precipitation from sodium chloride solutions and from ammonium sulphate solutions were identical.

Results of a preliminary electrophoretic survey of several plant proteins suggested that an electrophoretic study of the proteins extracted from pea meal would be an effective way of checking Osborne's conclusions. Solubility of these proteins is high, and fractionation by progressive precipitation is readily carried out. It was therefore decided to study the electrostatic properties of the protein material from pea meal in both the unfractionated condition and as fractionated by Osborne and Harris.

### Material and Methods

The peas used throughout this study were from a commercial sample of the variety Early Blue, grown in western Alberta.

The peas were ground in a Wiley mill and the meal obtained reground in a ball mill for six hours. The reground meal was extracted with ether in a Soxhlet apparatus for 48 hr. After drying, the extracted meal was sifted through a 12xx silk screen.

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Extraction of the meal was carried out using 0.2 *N* phosphate buffer of pH 7.8 for six hours. The murky extract was put through a Sharples super-centrifuge to remove solids and large aggregates. This clarified extract is referred to as the "unfractionated extract".

The first fraction was prepared by diluting this extract by the addition of 10 volumes of distilled water and adjusting the dilution to pH 5.2 with 0.1 *N* hydrochloric acid. The dilution was stored overnight in a refrigerator at 3° C., then centrifuged to remove the precipitate. The precipitate was taken up in 0.2 *N* phosphate buffer, and the solution then dialyzed against 1500 cc. of buffer for approximately four days. This preparation is designated Fraction A1.

The remaining solution was made 60% saturated with respect to ammonium sulphate, and adjusted to a pH of 5.2. The precipitate was removed by centrifuging, and prepared for electrophoretic determinations in a manner similar to that used with Fraction A1. This fraction is designated A2.

The solution remaining after precipitation of Fraction A2 was saturated with ammonium sulphate, and the above procedure repeated. The redissolved precipitate is designated Fraction A3.

A second extract was prepared and fractionation carried out omitting the dilution step. The clarified extract was made 60% saturated with respect to ammonium sulphate at pH 5.2. The precipitate was taken up in 0.2 *N* phosphate buffer. This fraction is designated as B2. The remaining solution was then made up to saturation with ammonium sulphate and the resulting precipitate prepared as usual. This preparation is designated Fraction B3.

Total nitrogen was determined using the micro-Kjeldahl method.

All electrophoretic analyses were carried out in a Tiselius apparatus of the type described by Longworth (2, 3). Temperature was maintained at 2° C. and all analyses were made using a field strength of 4.1 to 4.2 volts per cm.

## Results

### *Extraction of Pea Meal*

It was found as a result of preliminary work that it was impossible to get complete extraction of the proteins present in pea meal using salt solutions as dispersing agents. The protein in the unfractionated extract represented approximately 65% of the total nitrogen of the peas. Part of the other 35% was nonprotein in nature and part was not extracted by the 0.2 *N* phosphate buffer. The results reported in this paper obviously refer only to the 65% of the total nitrogen that was in the unfractionated extract.

Fractions A1, A2, and A3, respectively, contained 56%, 24%, and 14% of the protein in the unfractionated extract. Thus it appears that only 6% of the protein was not precipitated during preparation of the fractions. Unfortunately the amount remaining in solution was not checked directly, and since considerable mechanical error is involved in the various stages of



preparation, these figures must be taken as approximations only. According to Osborne and Harris (5), much more protein should have remained in solution after saturation with ammonium sulphate.

### *Electrophoretic Analyses*

In general, duplicate determinations of electrophoretic patterns agreed very well. Since none of the preparations yielded patterns with clearly separated components, it was necessary to interpret the nonsymmetrical nature of the patterns as indicating different components that varied within themselves in electrostatic properties. Mobility is taken as the basic criterion for identifying similar components in the different fractions.

The diagrams for the descending boundaries obtained with the unfractionated extract and with each of the fractions are shown in Fig. 1. There are three distinguishable components in the unfractionated extract with the

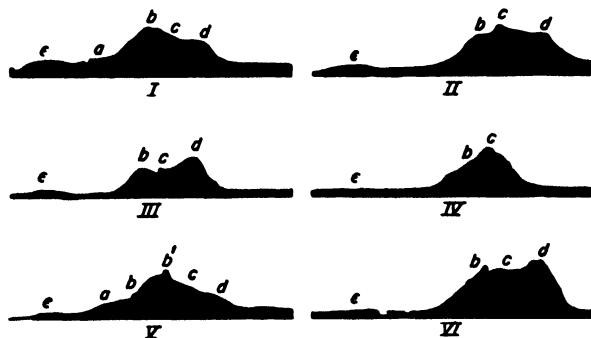


FIG. 1. *Electrophoretic diagrams; pH = 7.8, field strength = 4.1 to 4.2 volts per cm.*

- I. Unfractionated extract; protein conc. = 0.86%, time = 15,315 sec.*
- II. Fraction A1; protein conc. = 1.12%, time = 17,717 sec.*
- III. Fraction A2; protein conc. = 0.76%, time = 14,570 sec.*
- IV. Fraction A3; protein conc. = 0.76%, time = 18,418 sec.*
- V. Fraction B2; protein conc. = 1.04%, time = 16,973 sec.*
- VI. Fraction B3; protein conc. = 1.56%, time = 19,615 sec.*

suggestion that the middle one is actually made up of two. This is clearly shown in the studies of the fractions. The mobility of these components is included in Table I, which summarizes the mobility at pH 7.8, and the amount of each component in each fraction. The type of electrophoretic diagram obtained and the difficulty of satisfactorily separating the components make both the mobility values and proportions of each component subject to considerable error.

The mobility values are all negative, and while some variability is exhibited by the values for the same component in different fractions the identification is fairly definite. In earlier work with other more clearly defined components and in work with gluten protein (1) variability of the same order was experienced.

TABLE I

MOBILITY AND AMOUNT OF EACH PROTEIN COMPONENT IN THE VARIOUS PREPARATIONS

Fraction	Mobility, * 10 <sup>5</sup> cm. <sup>2</sup> /volt/sec.				% of total protein			
	Component				Component			
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
Unfractionated	2.19	4.50	—	6.14	9	61	—	29
Fraction A1	—	4.58	5.12	6.37	—	31	32	37
“ A2	—	4.13	5.03	6.45	—	28	15	56
“ A3	—	4.09	5.17	—	—	32	67	—
“ B2	2.14	4.32**	5.16	6.10	11	52**	20	17
“ B3	—	4.24	4.82	6.16	—	37	22	42

\* All mobilities are negative.

\*\* Fraction B2 appears to include another component (*b*<sup>1</sup>), with mobility of 3.82, making up 30% of the total protein. This component was reproducible in different runs.

The slowest component appeared only in the unfractionated extract and in Fraction B2. Actually B2 should contain the same components as A1 plus A2 unless the dilution step in preparation had a pronounced effect on the solubility of one or more of the components. There appears to be a marked effect on Component *a* since it was apparently not precipitated when dilution preceded salting out, but was precipitated when the dilution step was omitted. This component is probably an albumin-type protein, and may well correspond to the legumelin of Osborne (5).

Component *b* makes up a substantial part of each preparation, while *c* is clearly defined in each fraction. These two components are much alike in behavior, but the variability in proportions is difficult to explain. On the average they appear to make up about 60% of the total protein of the extract.

The appearance of the *b*<sup>1</sup> component in Fraction B2 was at first attributed to faulty technique. It appeared, however, to be reproducible. Thus, the precipitation by 60% saturated ammonium sulphate solution not only affected Component *a*, but apparently caused a separation of some of the other components. It seems not unlikely that the whole system represented by Components *b*<sup>1</sup>, *b*, and *c* is a typical Sørensen protein complex (6), with gradual variation in properties of the components. It also seems probable that the method of preparation affects the separation of these components under the influence of an electric current.

Component *d* is a fast-moving component and is clearly present in all preparations except A3. The difference between A3 and B3 must again be attributed to the effect of the preliminary dilution step in the preparation of the A fractions. It is not at all clear why Component *d* should be absent from A3 and yet be the largest component in B3. In any case, there seems to be no doubt that *d* is a clear-cut component distinguishable from all others, and accounting for approximately 30% of the protein in the pea meal extract.

In only one fraction does there seem to be good separation of components. Fraction A3 is made up wholly of Components *b* and *c*, which, as already suggested, may be considered to be components of a single system. The specificity of solubility is not high, however, since these components are not clearly separated from *d* in the preparation of Fraction B3.

In the preparation of the fractions discussed above, the solution from which precipitation occurred was always adjusted to a pH of 5.2. The pH of the precipitation medium is likely to affect the amounts and proportions of the different components precipitated, so an experiment was conducted to test this effect. Four preparations were used and treatment was identical except for the pH to which the extract was adjusted at the time of precipitation. The four pH levels used were 6.75, 5.50, 5.20, and 4.20. Ammonium sulphate was added to make the solution 60% saturated, and the precipitates prepared are for the other fractions.

Electrophoretic diagrams of the four preparations are given in Fig. 2. All runs were made at pH 7.70  $\pm$  0.06, while protein concentration varied from

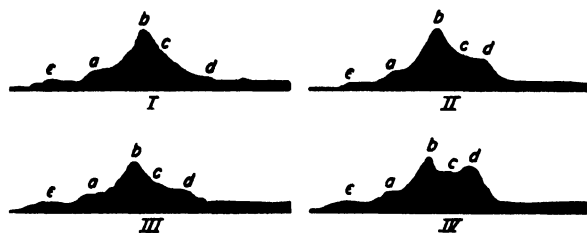


FIG. 2. Electrophoretic diagrams of pea protein precipitated at 60% of saturation with ammonium sulphate; pH at time of analysis = 7.8; field strength = 4.1 to 4.2 volts per cm.; protein concentration approx. = 1%; time approx. = 16,000 sec.; pH at time of precipitation: I = 6.75, II = 5.50, III = 5.20, IV = 4.20.

0.83 to 1.10%. The mobility values and proportion of each component are given in Table II. The preparation carried out at pH 5.2 should yield results

TABLE II

MOBILITY AND AMOUNT OF EACH PROTEIN COMPONENT AS AFFECTED BY  
pH OF THE PRECIPITATION MEDIUM

pH at precipitation	Mobility, * 10 <sup>8</sup> cm. <sup>2</sup> /volt/sec.				% of total protein			
	Component				Component			
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
6.75	2.33	4.11	5.26	—	14	62	16	6
5.50	2.19	4.15	5.30	6.46	10	51	22	17
5.20	2.01	3.90	5.11	6.02	14	55	17	16
4.20	2.12	4.01	5.14	6.18	11	40	20	30

\* All mobilities are negative.

in agreement with those for Fraction B2. Within acceptable limits of error this agreement is attained, if Components  $b$  and  $b^1$  of Fraction B2 are considered to correspond to Component  $b$  in Fig. 2. The low mobility for B suggests that this is really a mean value for  $b^1$  and  $b$ . There is no evidence of  $b^1$  in the preparations precipitated at 6.75 or 5.50, but more than a suggestion of this component in the other two diagrams.

Electrophoretic separation of the three Components  $b$ ,  $c$ , and  $d$  is progressively improved as the pH of the precipitation medium decreases. Component  $a$  seems to be entirely insensitive to this treatment; the amount of  $c$  seems to remain about constant; but the amount of  $d$  increases progressively while that of  $b$  decreases as the pH decreases. The determination of the basic cause of these changes will require further study, but there seems to be no doubt as to its importance.

### Discussion

The results presented here are preliminary in nature, and have already suggested many experiments that should be performed. They are sufficiently accurate, however, to add to the growing evidence showing the relatively poor separation of plant protein species that can be obtained by variable solubility alone. Since solubility has been used as the main criterion of protein classification, the importance of such evidence cannot be overstressed.

The components of the soluble proteins in pea meal are not clearly defined, but the results of this preliminary work show that distinct types are present, and that probably one of these is albumin in character. Whether the results are interpreted as indicating three or more components depends on the importance attached to the separation effected with the middle components in some preparations. Whatever the interpretation, however, the middle component or components appear to make up a typical Sørensen protein complex varying within itself in properties.

It is very unlikely that Osborne's methods (4, 5) yielded three distinct protein species. There is evidence that there are three different types of protein present in pea meal, but these are certainly not homogeneous, and the properties overlap sufficiently so that separation of types is not accomplished using precipitation methods. Electrophoretic separation of components in protein is much more precise than is any method of separation based on solubility (7). It seems certain, therefore, that Osborne's "three proteins" were mixtures of components, with the proportions of each component varying in the different fractions. Much more accurate work is necessary before the components can be adequately described.

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# PHYSICAL AND CHEMICAL PROPERTIES OF GLUTEN

## I. ESTIMATION OF MOLECULAR PROPERTIES USING ELECTROPHORETIC AND DIFFUSION DATA<sup>1</sup>

BY J. R. COLVIN<sup>2</sup> AND A. G. MCCALLA<sup>3</sup>

### Abstract

The molecular characteristics of gluten in sodium salicylate solutions have been studied by means of diffusion and electrophoretic techniques. The results of the observations indicate that gluten in sodium salicylate is electrostatically homogeneous with a high mean negative valence of 34 and therefore a high surface charge density. The isoelectric point of gluten in this dispersing agent is below pH 4.0. This low value of the isoelectric point and the high valence of the protein molecule is probably due to selective adsorption of salicylate ions on gluten. The particles in the fraction of gluten molecularly dispersed in 0.5M sodium salicylate are prolate ellipsoids of revolution of the order of 25Å in diameter and 400 to 450Å long. These are mean values as the molecularly dispersed particles are not uniform in mass. When they are aggregated it is side by side association rather than end to end. The density of dehydrated gluten is 1.291 gm. per cc. Qualitative evidence is given for the view that the degree of hydration of gluten particles is probably not great.

### Introduction

The results of an extensive study on gluten in sodium salicylate solutions using ultracentrifugal and diffusion methods were published in 1942 by McCalla and Gralén (26). These results showed that in sodium salicylate solutions gluten existed in two states, molecularly dispersed and aggregated. There was, however, progressive continuous variation in the physical properties and dimensions of both the molecules and the aggregates. This proved conclusively that "gliadin" and "glutenin" did not exist as distinct entities and also gave strong support to the view that gluten is a reversible protein complex, as suggested by earlier work (27,44).

The study mentioned above proved the heterogeneity of gluten in sodium salicylate solutions as far as mass characteristics are concerned but no corresponding study of the electrical characteristics of gluten in sodium salicylate has been made.

Moreover, only a very limited amount of work has been done on gliadin, gluten, or related proteins in other solvents. Wood and Hardy (49) had shown that the physical properties of a gluten ball were a function of the electrical charge on the gluten micelles, which in turn depended on the hydrogen ion concentration of the surrounding medium. They proved that the gluten micelles had a positive charge in acid medium and a negative charge

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in alkali, as was to be expected. Moreover, they could find no difference in the behavior of gluten and of a sample of gliadin. On the basis of these results, they concluded that the physical state of gluten was largely determined by the potential difference between the protein and the dispersing fluid. Kemp (14) and Kemp and Rideal (15) have used the microelectrophoretic technique with gliadin adsorbed on quartz to estimate the isoelectric point of gliadin and to compare the surface charge densities of various flours. They found an isoelectric point of approximately 5.00 for gliadin but this value was inversely dependent on ionic strength. Despite extensive data on the adsorption of gliadin on quartz, few data were given on the surface electrical properties of gliadin itself.

The only study of gliadin using the Tiselius electrophoretic technique is that of Schwert, Putnam, and Briggs (39). They found that gliadin dispersed in acetate buffers was markedly heterogeneous. Two main fractions of widely different mobilities and isoelectric points were identified but these two fractions themselves could be subdivided into components. Because of this heterogeneity and also because of a strong asymmetry between patterns obtained in the two legs of the electrophoretic cell, these workers concluded that gliadin was a mixture of components with widely different surface charges interacting in a non-ionic manner.

While the present study was in progress, Scallet (37) published a report of a study on zein in aqueous alcohol using the Tiselius electrophoretic apparatus. As the result of multiple fractionations followed by electrophoretic analysis he concluded that zein was a typical association-dissociation system as described by Sørensen (42).

Since electrophoretic data naturally supplement ultracentrifugal and diffusion data, the present study was undertaken. It seemed logical to suppose that the known variation in the chemical composition of gluten fractions (27) would be reflected in their electrical behavior and information on such variability would lead to a better understanding of the mechanics of the gluten complex. Specifically, it was hoped to determine the number of components in gluten and the electrophoretic mobilities of the components. Later, this objective was broadened to include the charge density of the molecularly dispersed gluten particle. These data, with existing ultracentrifugal and diffusion values, might then allow an estimate of the size and degree of hydration of the molecule.

### Materials and Methods

The gluten used throughout this study was obtained from an unbleached high quality flour milled from hard red spring wheat. The ash content of this flour was 0.45% and the protein content, 14.2%. When baked by a standard malt-phosphate-bromate formula this flour gave high quality loaves of good volume and texture. The gluten itself was in every way typical of that from the best Canadian spring wheat, being firm, elastic, and extensible.

All sodium salicylate used was of C.P. reagent grade. Unless otherwise stated, all solutions were of 0.5 ionic strength ( $0.5\mu$ ), which corresponds to slightly less than 8% sodium salicylate by weight. All preparations were checked before use against a standard solution by a conductivity apparatus.

Because McCalla and Gralén had found that gluten dispersed in sodium salicylate contained both molecularly dispersed protein and aggregates, the electrophoretic properties of two gluten preparations were investigated, i.e. molecularly dispersed gluten and whole gluten. Each preparation was represented by four samples independently prepared from the flour in as uniform a manner as possible and stored under constant conditions. The molecularly dispersed series was prepared as described by McCalla and Gralén for their CIV fraction except that the final dispersion was made in  $0.5\mu$  solution rather than in 12% sodium salicylate. This fraction is that portion of the gluten protein that remains in solution in 8% sodium salicylate made up to 8% of saturation with magnesium sulphate, but is precipitated when the concentration of magnesium sulphate is raised to 20% of saturation. Each of the samples in the "whole gluten" series was prepared by washing the starch from 100 gm. of ether-extracted flour with Dill and Alsberg's buffer (7) for 10 min. The resulting gluten ball was immediately torn into small pieces and allowed to disperse in a liter of sodium salicylate solution for three days with occasional gentle shaking. The undispersed gluten was then removed by centrifuging and the opaque solution clarified by adding magnesium sulphate until the solution was 1.5% saturated with respect to this salt. After removal of the precipitate by a second centrifugation, the solution was diluted to a protein content of from 0.6% to 0.8% and stored at 5° C. until used.

Electrophoretic mobilities and patterns were obtained at  $2.0^\circ \pm 0.05$  using a Tiselius apparatus described by Longworth (21). Details of dialysis of sample, cell manipulation, and instrument handling were essentially as given in papers by Longworth (19, 21), Longworth and MacInnes (24, 25), and Svensson (46) except for the following modifications. It was found that the power supply using a No. 80 rectifier tube could not maintain a constant voltage at the relatively high currents needed (20 to 25 ma.). Accordingly, B batteries in series were substituted as a source of potential. This arrangement held the voltage satisfactorily constant. Longworth (20) has described the use of a cell that eliminates errors in mobility due to movement of the rubber sleeves on the original Tiselius apparatus. Since no Longworth cell was available, it was necessary to use a Tiselius cell. This necessity has been reflected in the lack of precision in the pH-mobility curves (see Fig. 3) and is the greatest single source of error in this study. It was accentuated by the low mobility of the gluten in the buffer.

Mobilities were calculated using the method of Longworth and MacInnes (25) for locating the boundary and the formula  $\mu = \frac{Vk}{it}$ . Since no micro-comparator was available, the negatives were enlarged approximately four times and measurements were made directly on these enlargements. By this



method, boundaries could be located within 0.01 cm., which with a boundary travel of 1.5 cm., represents an error of 0.6%. This is well within the limits of error set by the source mentioned above.

Conductivities were measured using an Industrial instrument, which on testing with a calibrated cell showed an accuracy of  $\pm 1\%$ . This comparatively low order of accuracy had to be used since no better instrument was immediately available. The relative error due to this factor was probably reduced by the fact that the conductivity of the  $0.5\mu$  solution was quite constant at the given temperature for different samples.

Hydrogen ion concentrations were measured with a Beckmann Model G pH meter to  $\pm 0.05$  pH units.

It will be noticed that no buffer salts were used in addition to the main electrolyte. This was because repeated preliminary tests showed that no changes in hydrogen ion concentration took place in the boundary area even after 10 hr. of passing a 25 ma. current.

During the course of the study it was found necessary to determine the diffusion constant of molecularly dispersed gluten at  $2.0^\circ \pm 0.05$  in sodium salicylate. This was done by supporting a Neurath cell (30) in the thermostat of the electrophoresis apparatus at a position where the magnification was unity and using the formula  $D = \frac{A^2}{4 \pi t (I_m)^2}$  for calculation from the patterns obtained, as used by Rothen (36). With a polydisperse substance such as gluten, this formula yields only a mean value for the diffusion constant but this was satisfactory for a first approximation. A test of the method on 0.1 M sucrose at  $20^\circ$  gave a mean diffusion constant from 12 determinations of  $(44.3 \pm 0.9) \times 10^{-7}$  moles/cm.<sup>2</sup>/sec. as compared with the value from International Critical Tables of  $(44 \pm 2) \times 10^{-7}$  moles/cm.<sup>2</sup>/sec.

Viscosities were determined using an Ostwald viscosimeter calibrated against water at  $2.0^\circ \pm 0.05$ . Techniques were as described by Findlay (8).

After this investigation was nearly complete, it became apparent that it would be useful to know the density of dry gluten. No estimate of this constant could be found so an attempt was made to determine it by the methods of Neurath and Bull (31) for egg albumin. Their materials and technique were followed closely throughout except in the preparation of the sample.

As a means of estimating the relative importance of starch and electrolyte contamination in the final product, the gluten for the density determination was prepared by two methods each represented by five independent samples.

In Method 1, the gluten ball as obtained from 20 gm. of ether-extracted flour was merely washed extremely thoroughly with distilled water until only the faintest traces of starch were apparent in a sedimentation test. In general, this required more than an hour per sample. The thoroughly washed gluten was then frozen and, while frozen, ground to a coarse powder. The water was removed from this frozen gluten by lyophilization in a high vacuum over

$P_2O_5$ . Throughout this process, the sample was kept frozen by solid  $CO_2$ . The resulting coarse granules, which retained the natural color of gluten, were powdered in a mortar and stored over  $P_2O_5$  until used. The original properties of the gluten could be recovered by the addition of water to this powder.

In Method 2, the gluten was washed from 20 gm. of ether-extracted flour in the ordinary manner and dispersed for three days in sodium salicylate. The undispersed material was then centrifuged off and the opaque solution, now considered to be free from starch, made up to 20% saturation with magnesium sulphate. The gluten precipitated by the magnesium sulphate was collected on a stirring rod and washed as thoroughly as possible with distilled water to remove the electrolytes. It was then frozen and lyophilized in the same manner as for the undispersed series.

Throughout this study, all protein concentrations were estimated by a micro-Kjeldahl apparatus accurate to  $\pm 1\%$  of the total nitrogen present.

## Experimental Results

### *Preliminary Experiments on Egg White Proteins*

Before undertaking work on gluten, it was thought advisable to check apparatus and technique by duplicating previous work on a well-known protein. For this purpose, the work of Tiselius on egg albumin (47) was taken as reference except that the egg albumin was prepared according to the method of Kekwick and Cannan (13). The isoelectric point of the present preparation of egg albumin was found to be shifted 0.15 pH units to the acid side as compared with that of Tiselius (Fig. 1), which is three times greater than the

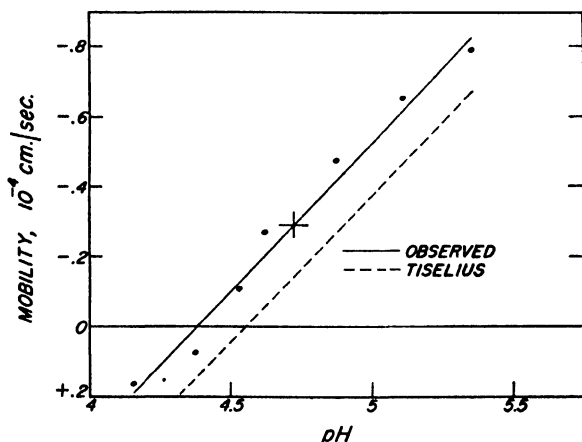


FIG. 1. A comparison of the observed pH mobility curve for egg albumin in 0.02 M acetate buffer at 20° C., with that of Tiselius for the same protein under the same conditions.

error involved in reading the pH meter. Moreover, tests showed this discrepancy to be reproducible. In spite of this, the results were taken as indicating the general reliability of our methods because the slopes of both

curves were identical. This could only be so if there were no serious errors in methods of observation and calculation. It was assumed that the difference in isoelectric points was due to small differences in the original material or to differences in preparation. It is unlikely that the difference is due to errors in the standard buffer solution because this was made up according to Clark (6) and checked against commercial tablets.

Since sodium salicylate is a denaturing agent for some proteins (4) and must be used in rather high concentrations for gluten, the possibility of its altering or obscuring some of the properties of the protein must be borne in mind. Rose and Cook (35) have shown that this reagent has only a slight effect on the properties of gluten as compared with the effects of the more usual dispersing agents, while Spencer and McCalla have demonstrated that there is no permanent alteration in the character of gluten after dispersion in salicylate (44). This does not prove that there is not a temporary modification of the surface properties, particularly surface charge densities, while the gluten is dispersed. To demonstrate the effect of sodium salicylate solutions on a well characterized protein (23) the patterns of egg white in this reagent and in Sørensen's phosphate buffer, which is widely used as a buffering agent, were obtained. The patterns for this mixture of proteins are given at 0.1 *M* and at 0.5 *M* for both solutions in Fig. 2. An inspection of these patterns shows

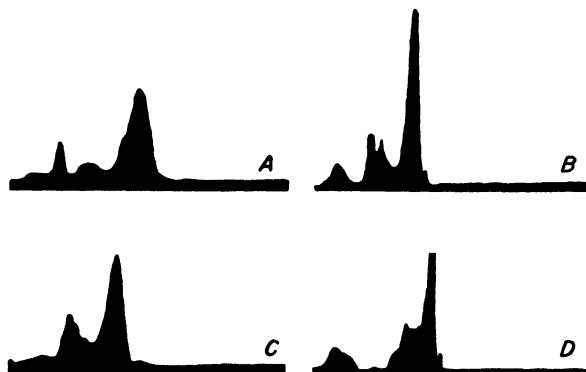


FIG. 2. Electrophoresis diagrams of egg white. Protein concentration = 1.00% in each test.

- A. In 0.1 *M* phosphate buffer at pH 6.9. Time = 60 min.
- B. In 0.1 *M* sodium salicylate at pH 6.9. Time = 60 min.
- C. In 0.5 *M* phosphate buffer at pH 7.0. Time = 200 min.
- D. In 0.5 *M* sodium salicylate at pH 7.0. Time = 330 min.

that the number of components of egg white in sodium salicylate is the same as that in phosphate buffer. Moreover, the separation of components in sodium salicylate is equally as sharp as that in phosphate. A comparison of the patterns at the two concentrations of both reagents indicates that the only effect of the increased ionic strength is a reduction in mobility, as might be expected. From this it was concluded that, for the proteins of egg white, sodium salicylate is at least as satisfactory a buffer as Sørensen's phosphate.

Unfortunately, a similar direct comparison of the two solutions for gluten is not possible owing to the insolubility of gluten in most neutral solutions. The assumption has to be made that sodium salicylate has no more specific effects on gluten than on egg white proteins.

### *Main Electrophoresis Experiments*

Following the completion of the preliminary experiments and a number of trial runs, pH-mobility curves for both the whole gluten and the molecularly dispersed gluten were determined. It was, of course, possible to obtain electrophoretic patterns for each series at the same time. The results of this study

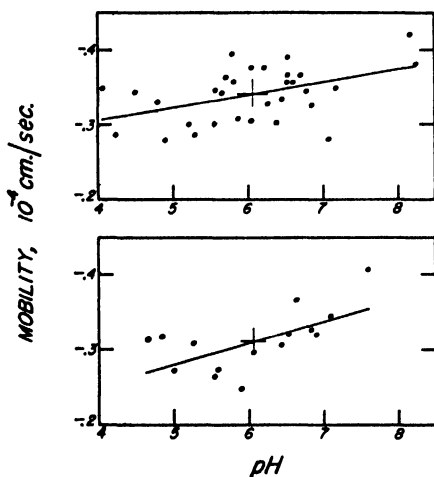


FIG. 3. Mobility of gluten dispersed in 0.5 M sodium salicylate as a function of pH.  
Top—whole gluten.  
Bottom—molecularly dispersed gluten.

are plotted in Fig. 3. Representative electrophoresis patterns for whole gluten and molecularly dispersed gluten are given in Fig. 4.

A study of Fig. 4 shows that both the whole gluten and the molecularly dispersed portion are electrically homogeneous in sodium salicylate. The patterns are asymmetrical to a small degree in some cases but no patterns were obtained in which a separation of the peak could not be correlated with an error in technique. The only conclusion possible is that in sodium salicylate all fractions of gluten are essentially the same electrostatically. This does not apply to all portions of the nitrogenous constituents of wheat, however. It will be noticed that the patterns exhibit a small peak migrating faster than the main boundary. This was a constant feature of all patterns and its presence is proof that if major components are present in gluten, this technique should discover them. That the small peak or boundary is not a major fraction of gluten is proved by its size alone; it represents only a very small fraction of

the total protein present and therefore cannot be important in the complete complex. It has been identified tentatively as an impurity in the sample due to the presence of nongluten protein (26).

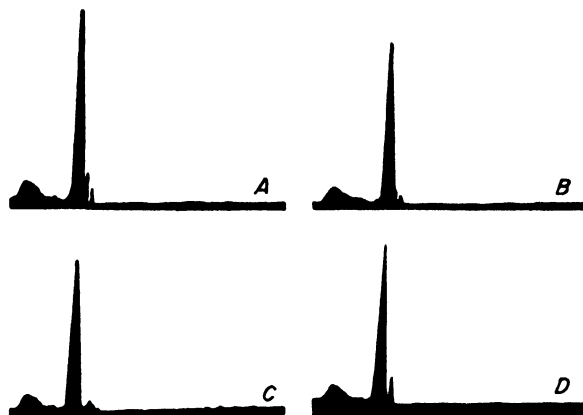


FIG. 4. Electrophoresis diagrams of gluten in 0.5 M sodium salicylate. Field strength = 2.3 volts per cm. Time = 250 min.

A. Whole gluten, protein conc. = 0.65%, pH = 6.3.

B. Whole gluten, protein conc. = 0.65%, pH = 7.2.

C. and D. Molecularly dispersed gluten, protein conc. = 0.5%, pH = 5.0.

The results in Fig. 3 show that within the pH range studied, 4.00 to 8.25, gluten carries a negative charge in sodium salicylate solution. Moreover, the size of this charge is remarkably insensitive to changes in pH. This is shown by the very low slope of the regression line. This low slope is probably due to the fact that it was not possible to work in the isoelectric range because salicylic acid precipitates from the solution at pH values less than 4.0. All pH-mobility curves approach zero slope at hydrogen ion concentrations remote from the isoelectric point. Independent evidence for the existence of an isoelectric point for gluten in sodium salicylate at a pH less than 4.0 was given by the strong tendency for the gluten to precipitate at pH values less than 5.0. Below pH 5.0 it was difficult to keep the gluten in solution although this tendency to settle out was not accompanied by the precipitation of salicylic acid until pH 4.0 was reached. This very low isoelectric point of gluten in sodium salicylate will be referred to again in connection with the high value of the surface charge density.

A comparison of the two sections of Fig. 3 shows that the slopes of the regression lines are approximately the same for whole gluten and for the molecularly dispersed fraction. This is confirmed by statistical analysis\* and this fact corroborates the suggestion from the electrophoresis patterns that the surface charge characteristics are the same in both cases, since they react similarly to changes in the medium. Statistical analysis also proves\*\*

\*  $t = 0.98$ ; D.f. = 41.

\*\*  $t = 2.36$ ; D.f. = 43.

that the mean mobility at any given pH is lower for the molecularly dispersed fraction than for whole gluten. Since the mean particle size is lower for the molecularly dispersed fraction (26) and the evidence given above indicates that the surface charge densities are approximately equal, this lower mobility must be a reflection of a larger frictional ratio  $\frac{f}{f_0}$ . This is in agreement with the observation of McCalla and Gralén that the most soluble fraction of gluten has the largest frictional coefficient. Assuming constant hydration, this in turn must mean that the particles making up the most soluble portion of gluten have the greatest length to breadth ratio.

These two experiments support two conclusions: 1. That in sodium salicylate solutions, gluten is an electrostatically homogeneous substance. 2. That the isoelectric point of gluten in sodium salicylate is somewhere below pH 4.0. Schwert, Putnam, and Briggs (39) obtained results on gliadin so much at variance with our conclusions concerning the nature of gluten that it was thought worthwhile to repeat their study using gluten from our flour. It proved possible to duplicate their work in all respects but one. Even at field strengths in excess of those mentioned in their report no multiple separation of the two main components into lesser complexes could be obtained. All other observations made by these authors were confirmed, however, proving that the source of the discrepancy does not lie in the material used. According to this evidence, the fundamental mechanisms of dispersion in acetic acid and in sodium salicylate must be different.

While this study was in progress, Scallet (37) published a report on the electrophoretic analysis of zein, in which he observed that as the ionic strength increased, separation of components in the mixture decreased. It has been suggested that this suppression is the cause of the apparent electrophoretic homogeneity of gluten in sodium salicylate. To test this point as far as physical conditions would permit, a dispersion of gluten in  $0.5\mu$  sodium salicylate was diluted until the protein was on the verge of precipitation. The final concentration of sodium salicylate was  $0.3\mu$ . An electrophoretic analysis was then made on the sample at this concentration. The results are shown in Fig. 5.



FIG. 5. *Electrophoretic diagrams of whole gluten in 0.3 M sodium salicylate. Field strength = 3.8 volts per cm., time = 250 min., protein conc. = 0.35%, pH = 6.0.*

It will be observed that this plate gives no more evidence for the heterogeneity of gluten than do those conducted at  $0.5\mu$ . One main peak only is present. Since  $0.3\mu$  is still a fairly concentrated solution and because the altered ionic strength may have changed the degree of molecular dispersion (26), this experiment is not conclusive but taken in conjunction with the presence of the nongluten protein boundaries, it is a strong indication that the homogeneity of gluten is not due to suppression by high salt concentration.

It was also thought that the failure to resolve components in gluten might be due simply to the low mobility in the high salt concentrations. If this were so, increasing the current-time product should tend to separate components. Accordingly, an experiment was conducted on whole gluten in  $0.5\mu$  sodium salicylate using as high a current as the thermostat would allow for as long a period as the electrode capacity would permit. The results are shown in Fig. 6.

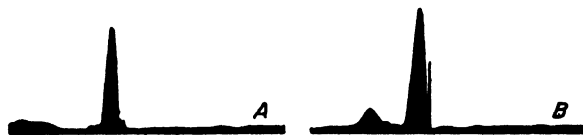


FIG. 6. Duplicate electrophoretic diagrams of whole gluten in  $0.5\text{ M}$  sodium salicylate. Field strength =  $1.7$  volts per cm., time =  $600$  min., protein conc. =  $0.5\%$ , pH =  $6.7$ .

These patterns are in every way comparable to those obtained under the less rigorous conditions and give no support to the above suggestion.

Summing up all the evidence, the most reasonable interpretation is that sodium salicylate does not seriously affect the separation of components. The work with egg white proteins, nongluten protein constituents, and with low concentrations of electrolytes supports this view. It must be pointed out, none the less, that this evidence is suggestive only, not conclusive. Sookne and Harris (43), working with the proteins of silk have shown that the more complex ions such as phthalate and picrate are preferentially adsorbed, sometimes to the point where the isoelectric point disappears from the range studied. If gluten constituents should selectively adsorb salicylate ions, it might raise their electronegativity to a point where smaller initial differences are not distinguishable. In fact, evidence of just such a mechanism is presented in the next section of this paper. It seems that a salicylate-gluten complex is formed, which is one step in the dispersion of the gluten mass. If this is so, it must mean that the surface characteristics of the gluten molecule or micelle are very different from those of egg white proteins and this in turn must be related to their respective amino acid compositions. That wide differences in the amino acid composition of the two proteins exist is well known (5) but as yet we know nothing definite as to how these differences may be reflected in the nature of the surface of the molecule. This question deserves much greater study.

### *Diffusion*

The values obtained from 13 determinations of the diffusion constant of molecularly dispersed gluten at  $2.0^\circ \pm 0.05$  are given in Table I. In the last column of this table, the diffusion constants are corrected to  $20.0^\circ\text{C}$ . in pure water in order to provide a comparison with previously published values (26) but for all calculations made in this paper, the diffusion constant is that for  $2.0^\circ$  in  $0.5\mu$  sodium salicylate. It will be observed that, in general, the

TABLE I

DIFFUSION CONSTANT OF MOLECULARLY DISPERSED GLUTEN AT 2.0° C.

Run No.	Time (min.)	$D \times 10^7$	D, corrected to 20° in pure water, $\times 10^7$
1	510	2.13	5.23
	1056	2.06	5.06
2	564	1.98	4.86
	762	1.89	4.64
	1333	1.97	4.83
3	518	2.13	5.23
	787	1.96	4.81
4	267	2.00	4.91
	503	1.97	4.83
5	248	2.28	5.60
	698	1.97	4.83
	1371	1.91	4.69
6	328	1.98	4.86

longer periods are associated with the lower diffusion constants. This is because some slight mixing of solutions is unavoidable when the boundaries are formed. The effect of mixing is to raise the apparent diffusion constant but it is obvious that the effect will approach zero as the time of diffusion approaches infinity. In order to approximate this condition the regression line of a plot of  $\frac{1}{t}$  against the diffusion constant was calculated and the "true" diffusion constant taken as the value of the intercept of the regression line on the diffusion axis. This value is thus an estimate of the diffusion constant of the material after a very long time has elapsed since the formation of the boundary. Corrected in this manner, the mean diffusion constant of molecularly dispersed gluten in 0.5μ sodium salicylate at 2.0° was estimated as  $(1.91 \pm 0.09) \times 10^{-7}$  moles/cm.<sup>2</sup>/sec.

It cannot be emphasized too strongly that the value of the diffusion constant obtained here is a mean value for the molecularly dispersed portion of gluten and therefore any particular part of this fraction may be expected to yield values that differ from the figure given. Since gluten is a polydisperse system (26), the same limitation applies to practically all numbers that are used to describe any appreciable part of the complex. These numbers are intended to give a reliable description of the protein, not to furnish exact magnitudes for a particular case.

#### *Density of Gluten*

The results of a determination of the density of dry gluten prepared by the two methods described previously are given in Table II. It is evident from



TABLE II  
DENSITY OF DRY GLUTEN AT 20° C., GM./CC.

Series	Sample	Density	Mean
Washed	1	1.299*	1.310
	2	1.312	
	3	1.311	
	4	1.310	
	5	1.309	
Reprecipitated	1	1.296	1.291
	2	1.292	
	3	1.288	
	4	1.291	
	5	1.288	

\*Rejected on a statistical basis according to Willard and Furman (48, p. 67).

inspection that the two series must differ and this opinion is sustained by statistical analysis.\* Since the washed samples were known to contain very small amounts of starch, which has a density of 1.50 (11), the mean of the values for the reprecipitated series was taken as the better estimate of the density of dry gluten.

### *Viscosity of Dispersions*

The values for viscosity of the dispersions of gluten in sodium salicylate as used in the other studies, and two values for the viscosity of 0.5 $\mu$  sodium salicylate as determined from independent solutions are given in Table III.

TABLE III  
VISCOSITIES OF DISPERSIONS, 2.0° C.

Series	Sample	Viscosity (centipoises)	Protein conc., %
0.5 $\mu$ Sodium salicylate	1	2.056	—
	2	2.054	—
Whole gluten	3	2.703	0.86
	4	2.482	0.87
	5	2.546	0.98
	6	2.461	0.84
Molecularly dispersed gluten	7	2.310	0.61
	8	2.312	0.62
	9	2.319	0.62
	10	2.320	0.61

Each value listed is the mean of at least five trials. Also listed are protein concentrations of the dispersions as calculated from mgm. of nitrogen per cc. These represent the limiting factor in the accuracy of these determinations.

\* $t = 10.9$ ;  $D.f. = 7$ .

There is, as pointed out by Abramson, Moyer, and Gorin (1, p. 137), a difficulty in the physical interpretation of viscosity as determined in the laboratory. In very dilute solutions of spherical proteins at their isoelectric point, the viscosity term occurring in the electrophoretic and diffusion equations "is clearly that of the medium and is equivalent to the coefficient of viscosity as determined in the laboratory". When the solutions become more concentrated and particularly when the particles are both highly charged and highly anisometric, this is no longer true. Instead of the protein molecule moving through a medium of pure dispersing agent, it now moves through a protein-solvent solution and the forces to which it is subjected may be appreciably different. This question has been discussed by Lauffer (17). He concludes from a study of the dependence of sedimentation constants, diffusion constants, and electrophoretic mobilities on concentration of the solution, that "there is considerable evidence that in the diffusion process and in electrophoretic migration a solute molecule must be considered to move through a medium with the viscosity of the solution rather than through one with the viscosity of the solvent". On the basis of this evidence then, the viscosity of the whole solution rather than that of the medium has been adopted as the closer approximation to the truth.

#### *Data for Electrical Charge on Gluten*

After several unsuccessful attempts to determine the charge on the gluten molecule by means of membrane potentials (2, 3) the moving boundary method as suggested by Longworth (22) and Svensson (46) was modified for use with gluten in sodium salicylate. Since Longworth had developed the theory in greater detail and checked it against other methods for egg albumin, his procedure was followed. Essentially, for a uni-univalent electrolyte, the application of this method depends on the determination of the relative mobilities of the protein, the buffer cation, and the buffer anion, together with the change in refractive index per equivalent of buffer and the change in refractive index at the buffer-concentration boundary (Longworth's nomenclature) per unit concentration of protein in the moving boundary.

Then, by application of the formula taken from Longworth (22)

$$\eta^{\beta} - \eta^{\gamma} = K_{AR} \left( \frac{r_B - r_R}{r_B} \cdot \frac{r_A}{r_A - r_R} - \frac{1}{2} \right) 10\rho e$$

where

$\eta^{\beta} - \eta^{\gamma}$  = change in refractive index at buffer-concentration boundary

$K_{AR}$  = change in refractive index per equivalent of electrolyte

$r_B$  = relative mobility of protein

$r_R$  = relative mobility of anion

$r_A$  = relative mobility of cation

$\rho$  = grams protein per 100 ml.

$e$  = charge in Faraday equivalents per gram of protein, after substitution of the appropriate constants, it is possible to calculate  $e$ . If the molecular weight of the protein is known, this allows an estimate of the

"valence" of the protein ion at the selected pH. Since most of these constants for sodium salicylate are not in the literature, it was necessary to determine them.

The  $K_{AR}$  value for sodium salicylate at  $2.0^\circ$  was estimated by an Abbé refractometer used on 10 samples of solution of progressively varying concentration. The value found for this constant was  $0.03417 \pm 0.00012$ .

The absolute mobility of the protein ion can be found from the electrophoretic measurements but in order to find its relative mobility it was necessary to know the mobilities of the sodium and salicylate ions at  $2.0^\circ$  in  $0.5\mu$  sodium salicylate. Such values are not given in the literature and the apparatus necessary for ascertaining them directly was not available. Accordingly, it was necessary to calculate values from data for these ions at infinite dilution and  $25^\circ$  (9, 10, 18) on the following assumptions:

1. That sodium salicylate is a strong electrolyte and completely dissociated.
2. That the transference numbers of the  $\text{Na}^+$  and salicylate ion are practically constant with variation in temperature.
3. That the transference numbers of  $\text{Na}^+$  and salicylate ion are affected only slightly by change in concentration.

Assumption 1 is probably true but it is well known that Assumptions 2 and 3 are not valid in general (28). Independent evidence for the validity of 3 in the case of sodium salicylate was obtained from the stationary character of the buffer concentration boundaries in routine electrophoresis experiments. These boundaries remain practically motionless over a period of four hours and this can only occur when the transference numbers of the ions are the same in both solutions (46). The correctness of Assumption 2 is more questionable but Getman and Daniels (9) and Glasstone (10) show that the variation is small, except for the most highly hydrated ions, over a wide temperature range. Therefore, for a first approximation and as a working hypothesis, it is believed that these two simplifications are justified. Using these assumptions and the published values for the absolute mobility of the sodium ion at  $25^\circ$  and infinite dilution (9) the relative mobilities of the three ions involved were calculated.

The determination of the change in refractive index at the buffer-concentration boundary per unit concentration of protein proved more difficult. The lack of a hollow prism made it impossible to apply Longsworth's procedure (21, 22). Moreover, because of the extremely small quantities involved, efforts to measure them directly failed. It was necessary to resort to an indirect method as follows: under the same conditions of temperature, magnification, and type of cell as in electrophoresis, large schlieren patterns due to a boundary between water and a  $0.1 M$  solution of sucrose in water were obtained. The method was essentially that used by Rothen (36) in his adaptation of the electrophoretic apparatus for studying diffusion of ribonuclease. Since the areas of these patterns as measured by a planimeter are

proportional to the differences in the refractive index of the two solutions at the boundary, which can be measured by a refractometer, it is possible to associate a unit area under a curve with a fixed difference in refractive index at the boundary (all other conditions remaining constant). Therefore, the area under a buffer-concentration boundary peak as obtained in routine measurements, multiplied by the constant obtained as above, gives the value of the refractive index change at the boundary. This change in refractive index can then be correlated with the protein concentration of the solution in the cell as determined by a Kjeldahl procedure. Since the relationship is linear for dilute solutions of proteins (22), the slope of this line gives the refractive index change per unit concentration of protein at the buffer-concentration boundary, which is the parameter required.

The experimental values obtained by the foregoing method are plotted in Fig. 7. The calculated regression equation is  $\Delta n = 10 \times 10^{-6} + 331.5 \times 10^{-6} \text{ mgm. N/cc.}$  with a standard error of estimate of  $17 \times 10^{-6}$ .

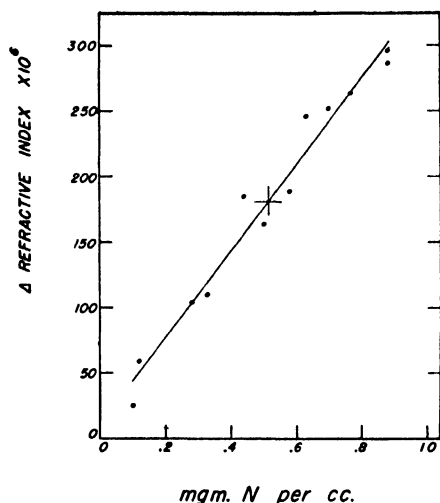


FIG. 7. Change in refractive index at the buffer concentration boundary as a function of nitrogen content of the solution.

Thus, within the limits of experimental error, the line passes through the origin as it should. The slope of this line is  $(331.5 \pm 18.5) \times 10^{-6}$  per mgm. N/cc., which, assuming a nitrogen factor for endosperm proteins of wheat of 5.70 (12), is equal to  $(581.6 \pm 32.5) \times 10^{-6}$  per gram of protein per 100 ml.

### Calculated Results

#### *Molecular Charge*

The foregoing experimental data permit the calculation of the mean charge of the molecularly dispersed gluten particles as outlined previously. The

constants used and the results of the application of Longworth's formula are summarized below:

DATA FOR CALCULATION OF MOLECULAR CHARGE

$$\Lambda_{(0.5\mu)} \text{ of sodium salicylate at } 25^\circ = 49.8$$

$$\Lambda_0 \text{ of salicylate ion at } 25^\circ = 37.1$$

$$\Lambda_0 \text{ of sodium ion at } 25^\circ = 50.1$$

Absolute mobility of sodium ion at infinite dilution and  $25^\circ = 5.2 \times 10^{-4}$  cm./sec./volt.

Ratio of conductivity of 0.5 $\mu$  sodium salicylate at  $2^\circ$  and  $25^\circ = 0.557$ .

$$\eta^{\beta} - \eta^{\gamma} = 331.5 \times 10^{-6}$$

$$\rho = 0.570 \text{ gm./100 ml.}$$

$$K_{AR} = 341.7 \times 10^{-4}$$

$$r_A = +1.000$$

$$r_R = -0.741$$

$$r_B = -0.188$$

Mean charge per molecule = 34 on the assumption that mean molecular weight is 44,000 (26).

This calculation gives the mean "valence" of molecularly dispersed gluten at pH 6.00 in 0.5 $\mu$  sodium salicylate as 34. From the low slope of the curve shown in Fig. 3 it is evident that this is an estimate of molecular charge over a considerable range of pH. For a protein of molecular weight 44,000, this valence is high. Egg albumin with a comparable molecular weight varies from 0 to -14 over a similar interval. This is a further indication of possible specific ion effects of salicylate on gluten (43). The reversible adsorption of negative salicylate ions on the gluten micelle may raise the electronegativity of the molecule to the observed high value. This would also explain the displacement of the isoelectric point so far towards the acid range and the observed flattening of the pH-mobility curve.

*Estimation of Molecular Weight from Diffusion and Viscosity Data*

It is possible to check the internal consistency of the data listed by a calculation of the molecular weight of the protein. Polson (33) and Svedberg and Pedersen (45) have shown that if the diffusion constant, the partial specific volume, and the frictional coefficient of a particle are known, a reasonably accurate estimate of the molecular weight is possible from the formula

$$\frac{f}{f_0} = \frac{RT}{D} \cdot \frac{(4 \pi N)^{\frac{1}{3}}}{6 \pi \eta N (3 m^v)^{\frac{1}{3}}}$$

$$\frac{f}{f_0} = \text{frictional coefficient}$$

$$R = \text{gas constant} = 8.313 \times 10^7$$

$$T = \text{temperature, } ^\circ K$$

$$D = \text{diffusion constant}$$

$$\eta = \text{viscosity of solution}$$

$$N = \text{Avogadro's number}$$

$$M = \text{molecular weight}$$

$$V = \text{partial specific volume.}$$

Using the mean value of  $\frac{f}{f_0}$  for the molecularly dispersed portion of gluten from McCalla and Gralén (26) and their value for  $V$  in 8% sodium salicylate (corrected for the difference in temperature according to Svedberg and Pedersen (45)) with the constants listed in the foregoing tables, a molecular weight of 48,000 was found for molecularly dispersed gluten. This is in satisfactory agreement with the ultracentrifugal value of 44,000 (26) and indicates the general reliability of the constants used.

These data also furnish reliable evidence against the hypothesis that gluten is a highly hydrated sphere. It is possible to explain the extremely large observed  $\frac{f}{f_0}$  value on the basis of a sphere with a thick shell of water. Using the value of the diffusion constant given and the formula

$$D = \frac{kT}{6 \pi \eta r}$$

where  $D$  = diffusion constant

$k$  = Boltzmann's constant

$T$  = temperature, °K

$\eta$  = viscosity of solution

$r$  = radius of particle

$r$  is found to be 45.6Å. From the density data given, an unhydrated gluten molecule of molecular weight 44,000 corresponds to a sphere of radius 23.8Å. Therefore in order to explain the observed diffusion constant by hydration of a sphere, it is necessary to postulate the adsorption of a shell of water approximately 20Å in thickness. Such a shell is extremely improbable on the basis of electrostatic forces alone (1, p. 151) and therefore the data must be interpreted on the assumption of rather large asymmetry of the molecule. Such asymmetry may be represented by two models: (a) the oblate ellipsoid, (b) the prolate ellipsoid. Simha has shown that for proteins in general and for gliadin in particular, the prolate ellipsoid is the much more probable model (41). On the basis of this evidence, therefore, it is concluded that an elongated ellipsoid of revolution is a reasonable approximation to the shape of the gluten molecule.

#### *Molecular Length-Breadth Ratio*

The frictional coefficient  $\frac{f}{f_0}$  has sometimes been used as a parameter for the estimation of the axial ratios of molecules. This procedure is not valid in general since the coefficient is a function of the degree of hydration of the molecule as well as its asymmetry. The two effects cannot be separated by a single measurement except for the special cases of zero hydration or completely spherical molecules (45). Polson (33), and more recently, Simha (40) have given equations from which an estimate of the axial ratios of anisometric molecules can be obtained from viscosity data. Polson's equation is an

empirical generalization of Kuhn's equation (16) for rod shaped particles and he has shown that it gives results in good agreement with experimental values. Simha's equation is an extension of Einstein's relation

$$\frac{u - u_0}{u_0} = v c .$$

where  $u$  = viscosity of solution                       $u_0$  = viscosity of solvent  
 $c$  = volume fraction of solute                       $v$  = is a shape factor

to the case where the particles are prolate or oblate ellipsoids suspended in a medium. He, too, has demonstrated that results calculated on the basis of his equation are in good agreement with experiment (40).

Substituting the data from Table III in Polson's equation

$$\frac{u}{u_0} = 1 + 4.0cV + 0.098cVk^2$$

where  $c$  = concentration of protein in grams per cc.

$V$  = partial specific volume

$k$  = axial ratio =  $\frac{\text{length}}{\text{diameter}}$  of ellipsoid

gives a  $k$  value of 16.3 for molecularly dispersed gluten. Substitution of the same data in Simha's equation

$$\frac{u}{u_0} - 1 = cV \left[ \frac{k^2}{15 \left( \ln 2k - \frac{3}{2} \right)} + \frac{k^2}{5 \left( \ln 2k - \frac{1}{2} \right)} + \frac{14}{15} \right]$$

leads to a  $k$  value of 17.1. Thus the results using the two methods are in essentially good agreement. Taking the mean of the two estimates gives a mean axial ratio of 16.7 for the molecularly dispersed gluten particle.

While the good agreement of the results using these two equations is reassuring it must not be overstressed. It is to be expected that they would tend to the same value since both are based on similar assumptions; that is, on complete Brownian movement among rotational ellipsoids. As has been pointed out by Robinson (34) it is of paramount importance that the basis of these equations be realized. If complete Brownian movement does not exist and the particles are to some extent oriented, viscosity is reduced and therefore the apparent value of  $k$  is lowered. The tendency towards orientation is particularly strong in capillary viscosimeters with their high rates of shear. Moreover, orientation is much more probable with greatly anisometric particles. Therefore, the value 16.7 is to be regarded as a lower limit until it can be confirmed by improved methods.

In spite of these limitations, if Perrin's formula (32) for prolate ellipsoids is used to calculate the  $\frac{f}{f_0}$  corresponding to a  $k$  value of 16.7, we obtain a frictional coefficient of 1.86, which is in satisfactory agreement with McCalla and Gralén's figure of 1.92 after the effects of possible hydration are considered.

*Molecular Volume and Radius*

A combination of diffusion data and frictional coefficient permits an independent calculation of the molecular volume (1). Using the equation

$$\frac{f}{f_0} = \frac{kT}{D6\pi n V^{\frac{1}{3}}} \left(\frac{4\pi}{3}\right)^{\frac{1}{3}}$$

where  $k$  = Boltzmann's constant

$n$  = viscosity of solution

$V$  = molecular volume

$D$  = diffusion constant

and the constants listed, we obtain a mean molecular volume for the gluten particle of  $56 \times 10^3 \text{\AA}^3$ . Calculation of the molecular volume of dry gluten from the density data listed also gives a value of  $56 \times 10^3 \text{\AA}^3$ . This agreement must be regarded as partly fortuitous, however, since the gluten molecule is almost certainly hydrated to some extent. Nevertheless, the close approximation indicates strongly that the hydration of gluten cannot be great or it would be reflected in a much larger molecular volume as calculated from diffusion data.

Combining the data for molecular volume from diffusion and the estimate of the axial ratio of the gluten particle from viscosity yields a radius of  $9.3 \text{\AA}$  for the gluten molecule. For the reasons pointed out above, this figure is certainly a little low since the effects of hydration would tend to increase the radius. It is possible to get a second, independent estimate of the molecular radius from electrophoretic results. Abramson, Moyer, and Gorin (1, p. 128) give the equation for electrophoretic mobility of a randomly oriented long rod as

$$v = \frac{\sigma}{\eta} \left( \frac{1}{\kappa} + r_i \right) \chi$$

where  $v$  = electrophoretic mobility

$\sigma$  = charge density of particle

$\eta$  = viscosity of solution

$\kappa$  = Debye-Huckel parameter

$r_i$  = average radius of ions in the ion atmosphere around the particle

$\chi$  is a shape factor, which, for long rods, has the form

$$\chi = \left[ \frac{\frac{\kappa a}{(\kappa a + \kappa r_i)} \frac{K_0(\kappa a + \kappa r_i)}{K_1(\kappa a + \kappa r_i)} + \kappa a \ln \left( \frac{a + r_i}{a} \right)}{1 + \kappa r_i} \right] \left[ \frac{4}{F} \right]$$

where  $a$  = radius of rod

$K_0$  and  $K_1$  are Bessel functions

and  $F$  is a function of  $\kappa a, \kappa r_i$ .



If the assumption is made that a prolate ellipsoid of great asymmetry approximates a long rod, this equation yields a molecular radius of  $14\text{\AA}$ . In contrast to the diffusion data this value must certainly be too high since it corresponds to a molecular volume of several times that calculated from density data. Such a degree of hydration is extremely improbable for reasons outlined before. Moreover, the relatively good agreement between the observed value of  $\frac{f}{f_0}$  and that calculated from the axial ratio is direct evidence against such a large hydration. The discrepancy is certainly due to the rather wide limits of error in the original data and to deviations from the original assumptions of the Debye-Huckel theory of solutions upon which the equation is based. Solutions of  $0.5\mu$  ionic strength, even of uni-univalent electrolytes, are entering the region where no known laws are obeyed and therefore it is to be expected that the results will be approximate only.

Summarizing then, the mean radius of molecularly dispersed gluten must be somewhere between  $10\text{\AA}$  and  $13\text{\AA}$ . With the present data, of a low order of accuracy, it is not possible to set closer limits.

### Discussion

These results serve to confirm and extend the concept of gluten as described by McCalla and Gralén (26). On the basis of ultracentrifuge and diffusion results they suggested that gluten was a reversible complex made up of molecules that varied progressively and continuously in both chemical and physical properties. On the observation of a high frictional ratio, the suggestion was made that these molecules were greatly elongated in shape but no data were presented to enable distinction between the possible alternatives of extremely high hydration of spheres or of a flattened shape. The enormous degree of hydration necessary to explain the diffusion values given in this study on the assumption of a spherical model, clearly exclude this possibility. Moreover, Simha, by his equation applied to viscosity data, has shown that the oblate ellipsoid model is extremely unlikely for gliadin (41). Since gliadin is a derivative of gluten, it is justifiable to extend his findings to the whole complex and so the prolate ellipsoid remains as the most likely model for the gluten molecule. In sodium salicylate solution, these ellipsoids of revolution are capable of existing singly or in aggregation, depending on the concentration of the salt in the solution and the protein fraction involved. If they are aggregated, the available evidence indicates that it is side by side association (26) rather than end to end attachment as in some viruses (38). The data given here indicate that the mean diameter of the particles making up the most soluble fraction of gluten is of the order of  $25\text{\AA}$  and their length of the order of  $400\text{\AA}$  to  $450\text{\AA}$ . This applies only to the molecularly dispersed fraction, of course. Very much larger aggregates undoubtedly exist in the ordinary dispersion. Again it must be emphasized that these are mean values, as the molecules, even in the molecularly dispersed fraction of gluten, are not homogeneous in mass.

It is interesting to compare the dimensions of the gluten molecule given above with those calculated for gliadin ten years ago by Neurath (29). Using Svedberg's estimates of 1.6 and 26,000 for the frictional coefficient and the molecular weight, respectively, of gliadin, Neurath found an axial ratio of 11.1 and a diameter of 18Å for the molecule of this derivative of gluten.

The mean surface charge density of gluten in sodium salicylate has a large, uniform, negative value that may be due to the reversible selective adsorption of the salicylate ions by the gluten. If this suggestion is valid it may offer a clue to the mechanism of dispersal of gluten in sodium salicylate. The bonds between salicylate ions and gluten particles may replace those between adjacent micelles, leading to the disintegration of the gluten mass. The sol so produced would then be stabilized by the high negative charge of the particles. Unfortunately, these data give no new information on the nature of the forces between gluten molecules or gluten aggregates. It was hoped that this method might suggest an explanation of the unique properties of gluten but this hope was unfulfilled.

In addition, these data give little new information on the extent of hydration of the gluten molecule. The observations recorded here are not of sufficient precision to permit a reliable calculation of the percentage hydration. Also, it seems doubtful whether the theory is sufficiently developed or the assumptions on which it is based sufficiently well fulfilled to justify the attempt at greater accuracy. In the writers' opinion, since the development of the lyophilization technique as applied to gluten removes many methodological barriers, a much more fruitful approach to the problem could be made by studying volume contraction. This has been used quite successfully by Neurath and Bull (31) on egg albumin.

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# PHYSIOLOGICAL AND BIOCHEMICAL STUDIES IN PLANT METABOLISM

## I. THE RESPIRATION OF THE SEEDLING WHEAT LEAF IN STARVATION AND ONTOGENY<sup>1</sup>

BY GEORGE H. DUFF<sup>2</sup> AND DOROTHY F. FORWARD<sup>3</sup>

### Abstract

Uniform conditions for the culture of the plants and for conducting starvation-respiration experiments upon the first seedling leaves permit the recapture, and hence thorough investigation, of transitory physiological states. A standard sample of isolated, mature leaves, so produced and starved, is shown to be heterogeneous when tested by the tempo at which the tissues in different parts of the sample pass through the color changes that accompany starvation. These are correlated with respiration. Interleaf variation in tempo is relatively small. Intraleaf variation is maximal and the leaf is polarized at isolation but a complex redistribution of tempo within each leaf brings about depolarization and diminishes intraleaf heterogeneity as starvation progresses. The respiration of such a starving sample follows a time course of characteristic pattern, all the prominent features of which have their homologues in the corresponding patterns of younger and older leaves. The homologous characters undergo gradual modification with ontogeny. The respiration of the unstarved isolated leaf is very high and falls rapidly during growth but is low and declines slowly in maturity and senescence. At the transition from growth to maturity a slight, temporary rise in respiration occurs.

### Introduction

In a wide area of the field of plant metabolism our knowledge is fragmentary because it has developed from studies of metabolic systems prominent in types of plants that are biologically remote from one another. The starch metabolism of the Solanaceae, the sugar metabolism of Monocotyledons, and the acid metabolism of succulent plants are familiar examples. Manifestly data have accumulated in this discrete manner for good reason and will therefore continue to do so. Nevertheless the circumstances invite attention to the advantage of a different approach, which is that of concentrating upon a single appropriate subject material an inquiry into as many aspects of its metabolism as possible. A series of researches instituted from this point of view has been and still is in progress in this laboratory. The results will be recorded in the present series of papers.

The experimental subject is the first foliage leaf of the seedling wheat plant, grown under uniform artificial conditions. It cannot be comprehended unless we take account of the succession of developmental stages through which it passes. From practical necessity we must also consider certain concurrently developing tissue and organ systems, but the main emphasis will be upon the leaf itself. It is typical enough of its kind and presents no marked peculiarities. It is exceptionally convenient in form and size and is adapted

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to the demands of the laboratory. An especially favorable property of the wheats is that they are readily cultured under artificial conditions (10). By so growing them the disturbing effect upon function of wide, unsystematic fluctuations in the conditions of growth can be minimized to the point of a rough standardization. For our purpose it is important to be able to return at will to a given physiological state or succession of states in order to examine separately as many aspects of these states as possible, yet always with the assurance that the data represent correlative attributes of the same state. Under favorable circumstances this advantage can be obtained for certain quantitative data within suitably narrow limits.

It is advisable to begin with the respiratory gaseous exchange because it is the most generally useful index of metabolic activity in the physiological organism. The experimental investigation of respiration in green tissues entails usually their isolation from the plant and always the imposition of darkness. If the observations are sufficiently long continued the tissues starve. In these circumstances, respiration rate, observed under uniform external conditions, does not remain steady but changes systematically in such a manner as to trace out in time a sequential pattern that is characteristic of the subject (2, 3, 4, 5, 6, 9, 11). The present subject is no exception to this rule. These patterned changes in rate are only in part determined by the controllable conditions of the respiration experiment itself. The determinants equally include the obscure internal factors that are inherent in the particular constitution which the interacting controls of development have produced. Some factors exert secular, others periodic effects upon the progressively changing respiration rate, thus characteristically influencing the pattern.

We look upon this pattern as a partial or guarded statement of the organism's metabolic constitution that it is our business to elucidate. Accordingly one of our objects will be to discriminate the factors determining the pattern, characterize their effects, and investigate the mechanism of their action. A contribution toward this end has already been made by Krotkov (8). Inquiries of this type take the physiological organism as the point of departure and proceed by the analytical consideration of such data as apply. On the other hand, some of our investigations begin at the other end of the organic scale with the distinction and separation of the enzyme systems. The probability of these two oppositely directed but complementary approaches reaching the desired common ground would appear to be greater when both courses are followed with the same subject.

In the present paper we shall restrict ourselves to respiration rate measured almost exclusively as carbon dioxide emission. Two kinds of progression of such rates must be considered. One denotes the sequence of metabolic states through which the wheat leaf passes during its growth, maturity, and senescence—an essentially ontogenetic progression. The other is essentially a starvation progression, manifested when the isolated leaf is maintained in the dark until it disorganizes. It is convenient to delineate the

former from data for the latter progression and these are derived through starvation experiments performed on leaves at different stages of development. Accordingly the matter presented in this paper is assembled in four sections: (a) procedure; (b) the starvation progression of a sample of leaves isolated at the standard stage of maturity, with evidence of heterogeneity in the sample; (c) the effect of age upon the starvation progression; (d) the ontogenetic progression of respiration rate.

## Culture and Treatment of the Experimental Materials

### *Seed and Planting*

To the present we have worked chiefly though not exclusively with *Triticum compactum* Host. var. Little Club and *T. dicoccum* Schübler var. Khapli. The latter has lent itself to our purposes particularly well and has been employed more frequently than the former. A full description of the cultural treatment of these materials follows and will serve the purposes not only of this but also of the majority of presently forthcoming papers in this series.

The seeds are planted in a light soil, which we maintain as uniform as possible. After a standard mixing and screening the soil is dispensed in equal quantities into waxed tin cans provided with drainage. Forty seeds are set out in a standard pattern, covered with a measured quantity of finely screened soil firmly pressed down and overlaid by two circles of filter paper. The latter are removed when the coleoptiles appear. A measured volume of tap water brought to temperature is given and the containers are transferred to growing chambers having a capacity of 96 such containers.

The following external conditions are normally maintained: Range of dry-bulb air temperature, 21.7° to 22.4° C. in time at the position of the thermograph; about the same variation in space when lights are on, less when dark; mean in time and space 22.2° C. The relative humidity is under dew-point temperature control and hence varies systematically with the rapid cycle of dry bulb temperature variations in time, the mean being close to 55% of saturation at 22.2° C. The light is derived from eight tungsten filament lamps per chamber each of 500 w. The light passes through a one-half inch water screen over glass and gives about 750 ft-c. at the soil surface. The light periodicity is 12 hr. alternate light and dark. Air is circulated through the chamber at 2500 c.f.m. and is wasted and replaced at the rate of 150 c.f.m. A measured daily ration of water is given to each container and this is augmented as growth develops and is diminished as sampling of the population proceeds.

### *Growth and Development*

Thus treated, the first coleoptiles of Khapli appear in a little more than 48 hours. At the end of three days the modal shoots are about 25 mm. long, the first leaf is entirely within the coleoptile, rolled up and not green in any part. A day later the first leaf is emerging, the terminal quarter of the blade is flat

and green, the second quarter green but rolled, the third quarter rolled and very pale green, the basal quarter rolled and shading in color from pale yellow-green distally to a colorless base. The blades are now about half grown and are 6 to 6.5 cm. long. During the fifth day the leaves expand to about 9 cm. in length but are otherwise generally similar to four-day leaves. By the end of the sixth day the first leaves are approaching full size, the second leaf is just emerging from the sheath of the first leaf, and the ligule of the first is only one-quarter of the length of the coleoptile from the top of the latter. The upper half of the leaf blade is mature in appearance, the third quarter is slightly rolled proximally and the basal quarter is rolled but green for three-quarters of its length, the remainder being pale green but not yellow-green. By the end of the seventh day modal first leaves are externally full grown and completely expanded with a length of 12 to 13 cm. The eight-day stage is our standard for the transitional or "adolescent" state in which the dimensional growth process is complete but residual internal changes are presumably in progress. The transitional period concludes on the 12th day. During this interval the plant becomes wholly autotrophic in its carbon nutrition.

On the 13th day the leaf quits the complex transitional state and enters the state of maturity. Our standard of maturity is the leaf on the 13th day from planting. Following this the external aspect of the leaf does not alter appreciably for about 10 days but shortly thereafter senescent changes become increasingly manifest in the terminal region and develop steadily until the tips of the leaves are yellowed and withered.

On the ninth day the rapidly developing second leaves are cut back in order to protect the first leaves from shading and for the sake of accessibility. Shoot growth continues but is cut back on alternate days so that the foliage of the seedling is restricted virtually to a single fully developed leaf.

Judging by the simple tests of relative size, form, color, and texture of the leaves the unnatural conditions of the growing chamber permit an adequate expression of the genetic characteristics of the wheats as they are normally manifested in the field or greenhouse. Several species and varieties have been grown and, depending upon the variety, the first leaves are from 25% to 35% longer at maturity than those of sister plants grown in the greenhouse. The green color is brilliant but slightly less deep than under good greenhouse conditions. The first leaves of the varieties maintain their characteristic differences of form, color, texture, and growth habit in the artificial just as in the more nearly natural environment. When such plants were allowed to develop without cutting back and grew to fructification under photoperiodicities of 15-9, 12-12, and 9-15, they fruited under all three, but fructification was accelerated by the augmented and retarded by the diminished photoperiod. The effect of the 12-12 photoperiod was deemed suitable for the purpose of developing a standard.

### *Selection and Sampling*

An unselected population of seedlings invariably contains retarded or abnormal individuals originating from broken or deteriorated seed which cannot be sorted out before planting if the seed carries hulls. Such individuals are clipped out at the surface of the soil as they appear. The remaining seedlings are not strictly homogeneous but represent an appreciable range of difference in size and in the tempo of development. Each population is therefore subjected to a selection designed to reduce the range of these variations. A series of measurements of length from tip to ligule is made on about 200 leaves taken at random on or immediately following the ninth day. The distribution of leaf length is charted and the modal length determined. Fig. 1 shows that the range of size is quite wide and the distribution normal. The entire population is then tested by the scale and only those individuals lying within 0.75 cm. on either side of the mode are normally retained. All others are clipped out of the containers without disturbing the root systems. With an average sample of unselected seed the yield of acceptable leaves is 20% to 25% of the number of seeds planted.

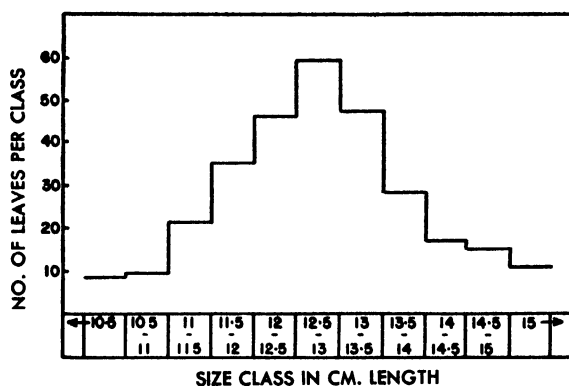


FIG. 1. *Distribution of leaf length in an unselected population of Khapli grown under standard conditions.*

The standard sample is composed of 50 leaves taken at random from the modal quartile of the population. At maturity in Khapli the sample weighs somewhat more than 5 gm. in the fresh condition and the leaves are 12 to 13 cm. long. The corresponding measures for Little Club are somewhat over 4 gm. in weight and 10 to 11 cm. in length. Such samples are convenient for most biochemical estimations as well as for our routine determinations of starvation respiration. Occasionally it has been advantageous to use larger samples as for instance, when the gaseous exchange must be measured over an unusually short interval of time or at very low temperature. Considerably smaller samples have necessarily been employed in studying the respiration of whole or divided leaves by micro-manometric methods. If close conformity to the standard conditions of growth has been successfully achieved the modal quartile will fall within the expected limits of size and



replicate samples of 50 modal leaves at maturity will yield routine respiratory data that agree to about 5%. Both elements of conformity are desirable in standard samples and normally both tests are realized. One or both may be disturbed by failure of control in the conditions of growth.

If the data are to be reproducible within sufficiently narrow limits, not only the conditions of growth but also the procedure for taking samples must be controlled. The starvation progression that we use as a general standard of comparison is that of the mature 13-day leaf. It is desirable that the general comparative standard should be a sample of relatively high sugar content and this we produce by giving 16 hr. of illumination instead of the normal 12 hr. on the day of sampling. The time at which sampling begins is determined by the number of samples to be taken simultaneously and is adjusted so that the manipulations are concluded shortly before the period of illumination ends. Handling the leaves as little and as gently as possible, they are cut at the ligule and stood in distilled water in groups of 50 in the growing chamber. When all the samples are complete they are rapidly weighed and returned to the chamber until the end of the 16-hr. period. They are then immediately removed to the laboratory and all are passed through the first steps of the further procedure within a very few minutes of zero time.

### *Respiration Determinations*

The samples for respiration determination are normally placed in cylindrical glass respiration chambers containing a very little water immediately after weighing and thus stand in the growing chamber until zero time. The respiration chambers are then closed, covered with tin foil, and placed in the thermostat. An air stream of 1500 ml. per hour is freed of carbon dioxide by being passed through a very large tower of soda lime and then through a long Pettenkofer tube containing clear barium hydroxide solution so arranged as to offer very slight resistance to flow. This is started up immediately and whenever the initial rate is of critical importance a preliminary run of 15 min. is allowed for clearing the system of existing carbon dioxide before estimations begin. Except where temperature effects are under examination, the thermostat temperature is identical with the mean temperature of growth ( $22.2^{\circ}\text{C}.$ ) and is uniform to less than  $0.1^{\circ}\text{C}.$  The evolved carbon dioxide is estimated by the Pettenkofer method adapted to continuous operation by an automatic air current commutator, which switches the air stream from one absorption tube to the next of a closed series, usually every three hours. Estimations are continued without interruption until tissue disorganization is evident. This requires about 115 hr. for the 13-day leaf at  $22.2^{\circ}\text{C}.$  The leaves are examined daily without opening the respiration chamber or stopping the air stream and the externally visible changes are recorded. The respiration rates are calculated as mgm. carbon dioxide per 100 gm. of fresh tissue per hour. Other methods of determining respiration must be used for special purposes

but since few such data are to be considered in this paper, these methods will not be referred to here except to note that the results by all the methods are reconcilable.

## The Starvation Progression at Maturity

### *Course of the Progression*

Fig. 2, A gives the respiration records of duplicate samples of mature Marquis wheat seedling leaves from a population grown in the greenhouse in July and isolated at the close of a long summer evening. These records are

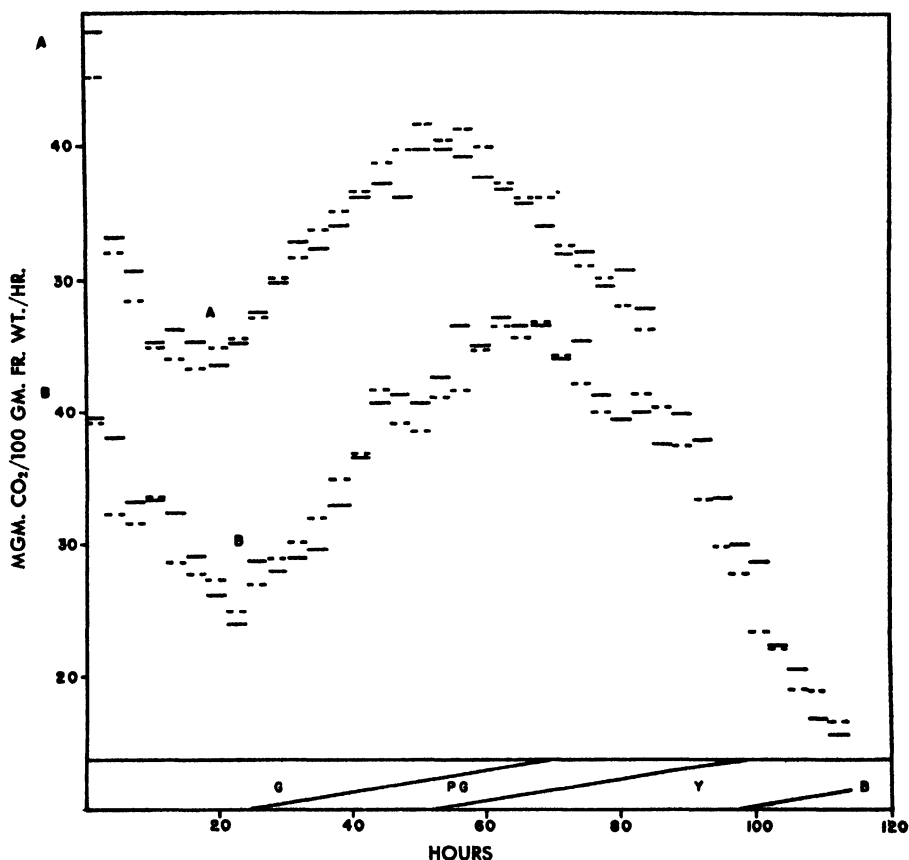


FIG. 2. Starvation respiration of mature first leaves of wheat seedlings.

A. Marquis grown in the greenhouse in July and isolated at dusk.

B. Khapli grown in plant chamber under standard conditions and isolated in full light. The diagram along the time axis indicates the changes of color that occur as starvation progresses. G—green; PG—pale green; Y—yellow; B—gray-brown.

presented first because they are devoid of certain complications that are present in the records of standard samples of Khapli and Little Club and that appear to be related to the sharp and exact photoperiodicity of the standard

conditions of growth. The complicating occurrences distort the form of the progression and though the undistorted form is deducible it is manifestly desirable to demonstrate it experimentally. Fig. 2, A presents the undistorted form as we observed it early in our work before any hypothesis of the origin and effects of the complicating factors had been developed.

The progression begins with a high but falling rate, which declines in a smooth down-valley curve to a transitory minimum. This is succeeded by a phase of almost uniformly accelerating respiration, which maximates and is followed by a phase of substantially uniform deceleration.

Fig. 2, B shows the course of the progression in duplicate standard samples of Khapli isolated on the 13th day from planting following 16 hr. of continuous illumination. The two samples were selected at random from amongst eight sister samples which had been taken simultaneously from the same population. Sample 1 happened to have the greatest fresh weight and Sample 2 the least but for one of the set of eight samples. The pair are a little less concordant in the early hours but rather more concordant in the later hours of starvation than the generality of well found duplicates.

The standard progression is evidently of the same three-phase form as that represented in Fig. 2, A, but in each of the three arms there here occur subsidiary inflections interrupting the smooth progression by either a pause or a temporary reversal of its sense. The first subsidiary inflection invariably occurs in all standard records, the second nearly always, but the third is in every respect more variable than the others and is frequently little or not at all developed. They are regarded as a superimposition upon the simple form of Fig. 2, A and, from the character of the distortion they produce in the simple form, the term "transposition" has been applied.

Accompanying the progression of respiration rates there proceeds a sequence of color changes in the leaf tissues that is similar to those described for other starving leaves and, like them, correlative to the respiration rate (6, 1). The original green color of the leaves has become perceptibly paler by hour 24 when the rising phase begins. The fading green passes into yellow that first appears more or less centrally in the leaf blades at about the 50th hour and progressively involves the whole blade except for very small amounts of tissue at the extremes, which tend to remain green. Later the color passes to gray-brown. The progress of these color changes is indicated in Fig. 2. This visible breakdown of chlorophyll denotes the concurrent progress of other deteriorative processes (6) which culminate in failure of semipermeability in the leaf cells and this results in the exudation of sap into the intercellular spaces. The injected appearance of the leaf blades that this event induces gives us a visible endpoint to the progression. Usually the respiration rate departs from the regular progression as this terminal stage supervenes and it may rise or fluctuate irregularly as autolysis rapidly involves more tissue. It is evident that at this point the physiological condition of the tissues has passed and the carbon dioxide evolved is no longer that of an organized respiratory metabolism. In the standard progression of Khapli leaves about 10% of the leaf

tissue becomes injected and respiration rate becomes irregular at or about hour 115 and these joint indications are taken to mark the end of the starvation progression.

The form of the progression evidently presents the same underlying characteristics as those of the mature leaves of durable physiological types that authors of the Cambridge school have considered (1, 2, 4, 6). The barley leaves which were the subject of Yemm's experiments (12, 13), though closer to our subject materials systematically, do not appear to show this form. We are therefore investigating a somewhat differently organized subject. The distinction from the durable types is prominently manifested in the time scale, which in the cherry laurel leaf is several times that of the wheat leaf and the denoted sequence of metabolic states is correspondingly well spread out in the one and foreshortened in the other. In the wheat leaf the progression covers a period of five days only and the successive somewhat overlapping phases pass by quick transitions from one to the next after a relatively brief duration. Though this fugitive quality imposes certain experimental limitations, the very abruptness of the transitions effectively calibrates the time scale in physiological dimensions and thus assists in the correlation of independently determined metabolic data with those for respiration.

The effect upon the progression of the constitutional difference between the durable types and the wheat leaf is also conspicuous in the minimum phase to which the descending first arm of the progression leads in them all. In the durable types the minimum may be much extended (2) and clearly denotes a relatively steady metabolic state that is distinct from preceding and succeeding states. In the wheat leaf the minimum seems by inspection manifestly transitional and this indication is confirmed in Krotkov's analysis of the progression (8) in terms of the ultimate substrate sources of the evolved carbon dioxide. Two components are recognized. One is identified with the first declining phase of the progression and its kinetics are held to be those of the metabolism of the sugar reserve. The other corresponds to the rise and fall of respiration that succeeds the minimum. The kinetics are complex and are dominated by the entry into down-grade metabolism of structural substances of the tissues of which the chloroplastic components stand merely as a type. These degenerative processes begin before the depletion of sugar is complete and, if these are the only components of the system, the overlap of the two changing rates, the one progressively diminishing and the other augmenting, must bring about the transitional minimum at the time when the two accelerations of opposite sign become numerically equal. In considering the two-component hypothesis we shall refer to the components as the depletion and the degeneration component respectively. Similar terms will be applied to the corresponding phases of the progression.

It is obvious that in so far as the time courses of the starvation progression of standard samples at the mature stage are congruent, the total carbon dioxide produced in the course of starvation must be uniform. The total carbon dioxide production by seven standard samples determined by ourselves

on Khapli 681 of three successive seed years, incidentally to work on the chemical constituents and not for the purpose of examining this datum in particular, ranged from 3928 to 4199 mgm. carbon dioxide per 100 gm. of leaves with a mean value of 4058 mgm. But though variations of limited range in the temperature of starvation exert a marked effect upon the time course of the progression, certain characteristics are not determinably influenced and amongst them the integrated carbon dioxide of starvation. Uniformity of carbon dioxide production therefore rests upon more than coincidence of rate along the time scale. An apparently similar relation has been observed in fruits (7). On the other hand the integrated carbon dioxide is strongly influenced by varying such conditions as lead to variation in the content of respiratory substrate at the time of isolation. The circumstances suggest a relation between substrate content and integrated carbon dioxide that will be considered in future papers.

### *Physiological Heterogeneity of the Standard Sample*

Before the conclusions from data for the standard sample can be formulated in terms of cellular function, we must take into account the physiological heterogeneity of the sample and consider both intraleaf and interleaf variations. The latter are the less problematical partly because they can be minimized through the method of culture, selection, and sampling. On the other hand there is no reason to believe, a priori, that the intraleaf variation can be correspondingly minimized by these means. An experimental inquiry into the magnitude and physiological controls of intraleaf heterogeneity is therefore inescapable and is begun though by no means concluded here.

Since the data at present under consideration are for sequences of metabolic state the element of heterogeneity that first attracts attention lies in the difference of speed at which the progression is passed through. This element we denote as "tempo". The standard sample displays both interleaf and intraleaf heterogeneity in tempo. Observations are readily made because, as noted in the preceding section, the progressive changes in respiration and metabolic state during starvation are accompanied by correlative changes in superficial color that can be observed in individual leaves and in the various parts of a single leaf. It is obvious that allowance must be made for a certain disparity arising from the fact that the observed color changes apply to the chlorenchyma tissues alone, whereas the data for respiration apply to other cell types as well though the latter are in small minority.

Our routine observations of the progressive color changes that occur during starvation show that the vast majority of the leaves undergo these changes so closely together that they cannot be distinguished as to tempo by this means. They also indicate that in the standard sample the intraleaf variation in tempo exceeds the interleaf variation sufficiently to appear as the definitive statistical element of the total heterogeneity. To define the heterogeneity more clearly we evaluated daily the color grade of each quarter of every leaf of a starving standard sample by matching the colors visually under uniform

conditions to a series of standards. These observations showed that the initial color grade of the unstarved sample was substantially uniform. After 24 hr. of starvation at 22.2° C., however, the sample by this test showed itself determinably heterogeneous in tempo, differences appearing both as between leaves and within the leaf.

Regarding first the interleaf variation, it was found that at the 24th hour about 85% of the leaves were indistinguishable as whole leaves. The variant 15% were not randomly distributed but were all laggards of slightly lower tempo than the large, modal group. This distribution cannot be assumed to persist throughout starvation but is probably complicated by changes entailing some increase in interleaf heterogeneity as starvation proceeds. But there is reason to believe that it applies to the first 36 hr. or more and our most critical data derive from this interval.

Taking the colors of the four quarters of the leaf separately as an index to the intraleaf heterogeneity at that time, the distribution of color grade at the 24th hour is shown in Fig. 3. It is evident that at this point the basal

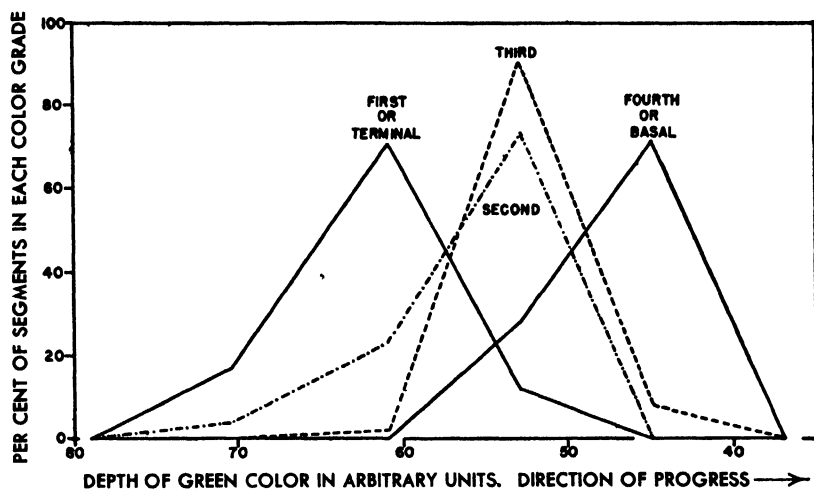


FIG. 3. Distribution of color depth in the four quarters (from tip to base) of a standard sample of intact Khapli leaves at the 24th hour of starvation.

region has the highest and the terminal region the lowest tempo. The two central quarters have identical modes but the distribution of the variants relates each of the central quarters to its neighbouring extremity so that a gradient exists though it is not uniform. This distribution of tempo does not persist but, as shown in the previous section, the central region overtakes the basal by the time yellowing commences at about hour 50. From this we must infer either that the earlier and later stages of chlorophyll degeneration are unrelated, which is improbable, or more probably, that tempo is redistributed as starvation progresses. Further light is shed on this question by the observations that follow.

When seedling plants are transferred to the dark and the first leaves starved without severance, a sequence of color changes occurs which is comparable to that of the isolated leaf except that it develops in a marked gradient and, at the same temperature, takes considerably longer to conclude. The terminal region is the first to yellow, the basal region is the last, and a gradient connects them. Evidently the attached leaf is characterized by a tip to base polarity in tempo which is maintained through the yellowing stages of starvation. As we shall show below, this is also the initial condition of the isolated leaf. Yet 24 hours of starvation after severance effects a complete reversal of the initial polarity and brings about the condition represented in Fig. 3. Then in the course of the next 24 hours the distribution changes further, bringing the central region instead of the basal into the most advanced position by the time yellowing begins. In all probability this complex redistribution proceeds continuously and apparently with a progressively diminishing amplitude of swing. It seems clear that its main statistical effect is twofold: In the first place it ultimately replaces a highly systematized or polarized distribution of tempo with a more nearly random or depolarized distribution. In the second place, the range of heterogeneity is diminished inasmuch as the time taken for the leaf to pass through the stages of chlorophyll degradation is considerably less in the isolated than in the attached leaf.

The factors in control of the initial polarity and those underlying the redistribution will be examined in future papers. In one of these it will be shown that when freshly isolated leaves are immediately cut transversely into quarters which are then starved concurrently but separately in a manner closely similar to that in which the standard sample is starved the terminal quarters strongly tend to turn yellow first and the basal quarters last with the central quarters intermediate. But the quarters themselves yellow first in their central parts. These effects of quartering demonstrate that the initial distribution of tempo in the leaves of an isolated standard sample is that of a gradient from tip to base and show that organic unity is a condition both of the initial polarity and of the subsequent depolarization.

Thus, judging by the limited physiological test of tempo, the chlorenchyma cell population of the attached leaf is marshalled in a polarized series and a maximal range of heterogeneity in tempo is maintained. We may suggest that this denotes a fully organized condition of the cell population and is a correlative of its role as a part of the intact organism. Upon severance such elements of organization as are contingent upon organic unity with the plant must necessarily be abated. What we observe under these circumstances is an abatement of the original polarity and of the original high degree of heterogeneity in the tempo of the starvation color changes. The characteristics of greater intraleaf homogeneity that develop as starvation progresses to the yellowing stage may thus be regarded as the insignia of a progressively lowering grade of organization. At the simplest, both the intercellular and intracellular aspects of organization will have to be considered. The former is evidently abated by isolation though presumably other factors are also influential.

The abatement of the latter is induced through starvation. It seems clear that the redistribution of heterogeneity in tempo must influence the sequence of metabolic events during starvation of the standard sample and thus cannot be disregarded in an analysis of the starvation progression.

There can be no doubt that the changes of color grade and respiration rate are correlative but the relation is clearest with the degeneration phase of the starvation progression. Observations have usually been made at the fairly advanced stage of yellowing and indicate that this stage is marked by increasing respiration rate (1, 2, 6). The relation between tempo and respiration in the earlier stages of starvation is not correspondingly clear and the phenomena accompanying depolarization suggest that it may be far from simple. The data of Fig. 4 show that under appropriate conditions the respiration rate in the initial stages of starvation has a polarized distribution like that of tempo. The data were derived from a group of 12 mature Little Club leaves which were quartered soon after gathering and the oxygen uptake of

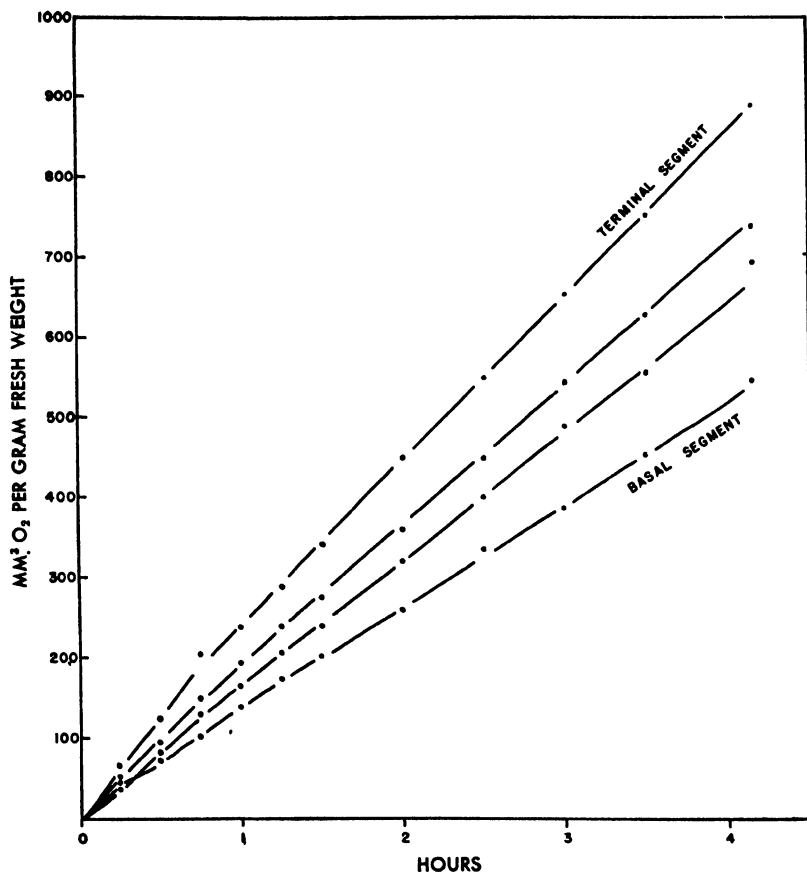


FIG. 4. Time progress of oxygen uptake in the four separated quarters of standard, mature leaves of Little Club wheat, showing a gradient of respiration from terminal quarter to basal quarter.



the composite four quarters determined manometrically. Calculated on the fresh weight basis a gradient in respiration is evident, but in a forthcoming paper a co-worker will show that the initial respiration gradient changes with ontogeny and is very susceptible to the influence of factors associated with modified water relations. The data of Fig. 4 therefore are not by themselves definitive. Nevertheless they demonstrate the reality and magnitude of the intraleaf heterogeneity in respiration rate.

### Effect of Age upon the Starvation Progression

Fig. 5 gives three records of Little Club cultured under standard conditions from seed grown at Moscow, Idaho, and treated experimentally as described in an earlier section. The samples were isolated on the 6th (A), 13th (B),

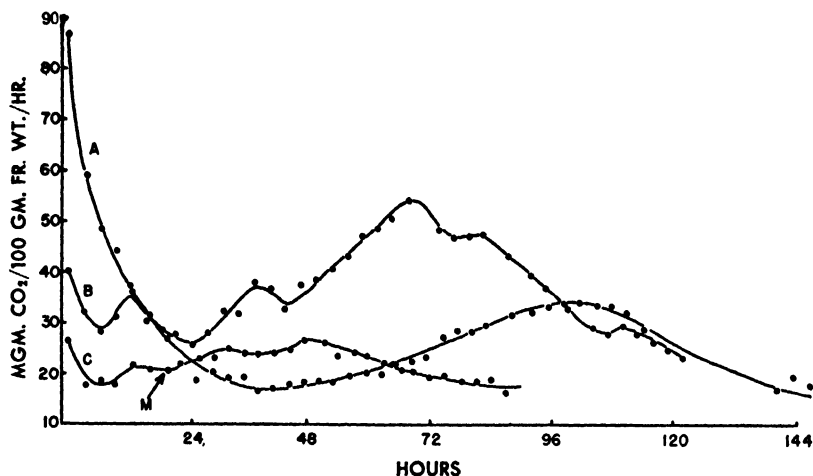


FIG. 5. Starvation respiration of standard leaves of Little Club isolated at three stages of ontogeny: A (juvenile), 5 days; B (mature), 13 days; C (senile), 40 days from planting.

and 40th (C) days from planting. Data for many intermediate stages have been derived but our immediate purposes can be served by the three examples. It is evident that the two extreme records are variants of the form typified by the standard of maturity (5B), which has already been considered. They must therefore present homologous characteristics, the comparison of which over the entire series would describe the effect of age upon the starvation progression. The more prominent of such characteristics are (a) the initial rate, (b) the duration of life and total carbon dioxide production, (c) the dimensions of the depletion phase and the minimum, (d) the dimensions of the degeneration phase, (e) the transpositions. Each of these manifestly undergoes modification with age. A thorough-going analysis must await the presentation of matter that cannot be recorded here, but the broad outlines of the changes occurring along the age scale will be formulated.

### *Initial Rate*

This attribute is clearly very high in young, growing leaves and falls off with age. The data of Fig. 5 show that the decline is very rapid during the premature and is much more gradual in the postmature stages. It is clear that initial rate must be considered in connection with the ontogenetic progression of respiration rate and we therefore reserve the further data for the following section of this paper.

### *Duration of the Progression*

The terminal condition of breakdown is reached in 6-day leaves in about 144 hr., in 13-day leaves in about 115 hr., and in 40-day leaves in about 87 hours. The duration of life in starvation thus contracts from youth through maturity to old age but most of this contraction occurs actually at the extremes of the age scale. The total production of carbon dioxide is manifestly much smaller in old age than at any earlier stage. The difference between the 6-day and 13-day leaf in this respect is in favor of the former but is much smaller than the difference between the 13-day and 40-day leaf. The prominent distinction between 6- and 13-day leaves lies in the distribution of the carbon dioxide production as between the depletion and degeneration phases.

### *Depletion Phase*

The very high initial rate of the young leaf is associated with the occurrence of a depletion phase of corresponding dimensions which requires 40 hr. to reach the transitional minimum. The decline of respiration rate in this phase is characteristically smooth and unbroken by a transposition with the result that the simple form of progression (cf. Fig. 2,A) is manifested in the growing leaf. The total carbon dioxide production of the depletion phase in premature leaves is substantially greater than that of any subsequent stage.

The depletion phase of the mature leaf, beginning with its much lower initial rate, requires 24 hr. to reach the transitional minimum and a much smaller amount of carbon dioxide is produced than in the premature leaf in this phase. The phase is interrupted in the instance before us by a particularly prominent transposition which so divides the phase that the simple form is much distorted and obscured. This transposition is always present but not always so strongly developed as in Fig. 5, B.

In the 40-day leaf further contraction in the dimensions of the depletion phase is manifest. The pitch of respiration is low and the carbon dioxide production correspondingly small. The extent of the phase in time is obscured by the transpositional rise and by the fact that the post-transpositional decline to the minimum is in this case so slight that the minimum could not be confidently identified by inspection. Because of the transpositions it has required a very close comparative study of several full series to decide the homologies of the much reduced respiration records of senescent standard leaves. These considerations will be presented in a following paper the conclusions of which we must here anticipate to the extent of identifying the point marked *M* in

Fig. 5, C as the homologue of the more evident minima of Figs. 5,A and 5,B. This identification leads to the conclusion that the extent of the depletion phase in time is but slightly less in the senile than it is in the mature leaf.

### *Degeneration Phase*

The onset of the degeneration phase, which begins with the transitional minimum, occurs progressively earlier during growth. During maturity and to a fairly advanced stage of senescence the degeneration phase is initiated under standard conditions with great regularity at the 24th hour from isolation and darkening of the leaves. This period becomes somewhat shortened as the leaves pass into the remote stages of senescence. The duration of the degenerative phase in 6-day leaves is somewhat over and in 13-day leaves is somewhat under 100 hr., but in the 40-day leaves the interval has declined to about 66 hr. The carbon dioxide production of the degenerative phase increases slightly in premature leaves with advance in age, the mature leaf having maximal production. During senescence the production declines at an increasing rate till in 40-day leaves it is about half that of mature leaves.

In all the leaves we have examined that were cultured and observed under standard conditions the degeneration phase is present and has the form of a rise to a maximum followed by decline. Sometimes in advanced stages the peak may be considerably flattened and the falling arm may then not decline to values as low as the minimum before the terminal disorganization supervenes. In the durable physiological type of leaf represented by the cherry laurel (6) the starvation progression in juvenile leaves is devoid of the rise and fall that characterizes the degeneration phase of mature leaves of this species and of wheat leaves of all ages. But the time scale of age in the wheat leaf is so short that the tissues of an isolated sample possibly make considerable progress ontogenetically during the course of a starvation experiment in which event observation of the penultimate stages of starvation with the tissues remaining in a juvenile state might be difficult.

### *Transpositions*

The occurrence of these inflections in the respiration records of mature leaves has been referred to in the preceding section. Fig. 5 shows that they are not present in the first declining arm of the record of the 6-day Little Club leaf. The early transpositions appear as the leaves cease growth and undergo the transition to maturity. In Khapli they (or their equivalent) appear somewhat earlier, in the 7-day leaf. Apparently the wheats differ in this respect. The number of juvenile records we have observed is far fewer than the number of mature records but they support the statement that the early transpositions are absent (or exceedingly weakly developed) in the records of growing leaves but are invariably present in those of all mature and postmature leaves cultured under standard conditions.

## The Ontogenetic Progression of Respiration Rate

The progression of respiration rate with age should ideally be delineated by some measure of respiration that has not been affected by depletion nor by any other variable factor except the age factor itself. The observed initial rate of the starvation progression complies substantially with the stipulation regarding depletion but not with the more general stipulation. It will be shown by a co-worker in a following paper that initial rate, as here derived, is in all probability affected by excitation of the leaves induced by the handling incidental to the preparation of the sample. Thus the initial rate is higher than that proper to the unexcited state. Also the excited initial state is not an adjusted or drifting steady state but is perturbed, and the perturbation lasts for several hours. These effects cannot be corrected for as yet, but in some instances they may be evaded by the adoption of another measure. It will be shown by methods adapted to following rapid changes in respiration rate that the perturbation passes with the beginning of the transposition and that the rate then settles down more or less briefly before the transpositional rise occurs. Though depletion is probably effective by this time, there is reason to believe that this could be allowed for. This adjusted transpositional rate might then be adopted uniformly as the measure of respiration for determining the ontogenetic progression were it not that the starvation progressions of young leaves lack the early transpositions. In the circumstances we present the ontogenetic progression over the whole age series in terms of initial rate and supplement these data with those for transpositional respiration rate in the mature and postmature stages where early transpositions invariably occur.

Numerical values of initial rate are given in Table I and plotted in Fig. 6 (A and B) for both wheats. Two populations of Khapli and one of Little Club provide the available *ad hoc* data. Supplementing these a great many observations of initial rate have been made incidentally, all of which confirm the essential features of the progression as here delineated. The populations were grown under the standard conditions described in Section 1 except that the dry bulb temperature varied beyond standard limits and the mean hourly temperature was a little below the standard. Also the plants were removed from the growing chambers to a dark room and back again each day. Since the frequent handling of the containers has a slight retarding effect upon development, the result of these two circumstances is to extend the time scale as here presented somewhat over that characteristic of strictly standard conditions. No other difference is observable.

Fig. 6 portrays the rapid decline of initial rate during the later growth stages of the leaf when stretching is the dominant phase of growth but not the only growth activity in progress. By the time dimensional growth has ceased (eight days, cf. Section 1) respiration is an order of magnitude lower and thereafter changes very much more slowly. The rates of the youngest observed leaves are so high and their decline is so steep that the question of the magnitude and course of respiration in the antecedent stages is raised. To shed

TABLE I  
ONTOGENETIC PROGRESSION OF RESPIRATION RATE

Days from planting	Initial respiration, mgm. CO <sub>2</sub> /100 gm./hr.			Transpositional respiration, mgm. CO <sub>2</sub> /100 gm./hr.	
	Little Club 8	Khapli 7	Khapli 8	Little Club 8	Khapli 8
5	111		99.5		
7	44.3		56.9		
9	37.0	43.6	41.5		
12		48.9			
13	40.5		42.9	31.5	32.0
19	31.6		37.4	25.0	30.0
22		37.1			
23	31.8		38.0	25.5	33.0
27	29.1		32.6	22.5	26.0
28		36.5			
32	28.1		30.4	22.2	23.5
35		26.6			
36	31.5		27.7	21.5	19.5
40	26.5			19.5	

some light on this question seeds of Little Club were germinated in moist chambers at 22.2° C. and, when the stage characteristic of about 48 hours' growth under standard conditions (soil) had been reached, the embryos were

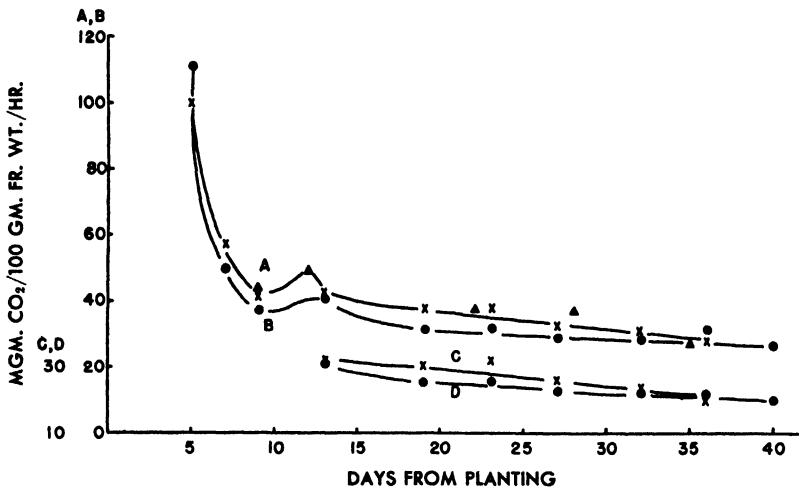


FIG. 6. Respiration of standard samples of Khapli and Little Club leaves isolated at various times during growth, maturity, and senescence.

A. Initial (unstarved) respiration of Khapli; crosses and triangles indicate the values derived from two populations grown on different occasions.

B. Initial (unstarved) respiration of Little Club derived from a population grown concurrently with one of Khapli.

C. Transpositional respiration of Khapli.

D. Transpositional respiration of Little Club.

Values plotted for C and D were both derived from the concurrently grown populations of A and B.

separated from the endosperms and their respiration separately determined at 22.2° C. after the manner of Barnell's experiments with barley (3). The initial rate proved to be of the order of 300 mgm. carbon dioxide per 100 gm. fresh germlings per hour. The exceedingly rapid decline in rate per unit mass of fresh tissue therefore probably continues for some time previous to the 5th day. Since stretching is in progress throughout this time in one part or another of the heterogeneous tissue systems, the enormous uptake of water that characterizes the stretching phase must account for a great part of the sharp decline in rate of carbon dioxide production per unit mass of fresh tissue. Since germination metabolism begins with low rates it is evident that the sharp decline in rate here observed must be preceded in a given tissue by an even sharper rise and at some early stage a maximum must be developed.

Between the 8th and 12th days the seedlings make the transition to a completely autotrophic carbon nutrition. Though dimensional growth is complete by the 9th day it is probable that some processes of structural differentiation are continuing in the leaf for the next day or two. During this interval also the vertical posture of the seedling leaf gives way to a lateral posture which must entail considerable internal differences arising from the incidence of radiation. The physiological state of the tissues is thus in all probability a complex one as the characteristics of juvenility fade out and are replaced by those of maturity. The data of Fig. 6 indicate that a slight, transitory rise in initial rate occurs during this interval. We were at first uncertain of the reality of this rise but confirmatory evidence of its reality in the case of Khapli will later be presented by co-workers. Accordingly, we incorporate the transitory rise as a formal characteristic of the ontogenetic progression when the progression is delineated in terms of initial rate.

These complications are normally past by the 13th day and because this is the earliest time of which this is true we have adopted the 13-day leaf as our standard of maturity. Owing to the slight departures of growth conditions from the narrow range of the standard, the 13-day leaves of the present series appear to be not quite clear of the transitory rise. Following the 13th day, however, it is evident that the initial rate gradually declines throughout maturity and into senescence. The values give evidence of increased variability after about the 30th day and this we have treated as a random scatter which presumably indicates an increasing heterogeneity of the population as progressive disorganization becomes important (cf. Section 1). In this connection the regularity of the data in the earlier stages may be remarked. This is especially manifest in the case of Khapli for which the data of two populations grown at different times from the same seed material are available. One population was grown and experimented upon concurrently with that of Little Club and the two sets of data therefore apply to strictly parallel material. The other population of Khapli was independently grown yet shows the same differences from Little Club. It is clear that while the ontogenetic progression is closely similar in the two wheats, a consistent, if

narrow, margin of numerical difference is demonstrable. The three starvation progressions for Little Club given in Fig. 5 were taken from the series which yielded the initial rates given in Fig. 6, B.

We may conclude by surveying the ontogenetic progression as far as it can be given in terms of transpositional respiration rates. These are also recorded in Table I and Fig. 6 (C and D). Where the transposition entails a definite rise in rate the median value between the extremes is taken, otherwise the transpositional value itself. The transpositions in Little Club always entailed a rise but this was true of Khapli in the 13-, 19-, and 36-day records only. In the other three Khapli records the transposition took the form of a pause in the decline of rate. As far as the data go it is clear that though the numerical values are lower than those for initial rate the progress in time is substantially the same. A possible exception to this is the slight rise that is suggested at about the 23rd day when the leaves begin to give external evidence of senescent changes (cf. Section 1). Fig. 6 shows that an overliteral interpretation of the numerical values for initial rate would lead to the same conclusion. With transpositional as with initial rates the two wheats maintain a similar, narrow margin of difference from one another. We conclude that as far as they can both be applied, the two respiratory measures give substantially the same account of the ontogenetic progression.

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The authors are indebted to Dr. C. S. Hanes for determining the oxygen uptakes given in Fig. 4, to Dr. Anne B. Brodie for the data on size distribution in a standard population given in Fig. 1, and to Dr. D. W. A. Roberts for the careful observations of growth progress recorded in Section 1 of this paper. They are obligated to the United States Department of Agriculture, the Dominion Rust Research Laboratory, Winnipeg, and University of Saskatchewan for contributions of seed.

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# Canadian Journal of Research

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## STUDIES OF CANADIAN THELEPHORACEAE

### III. SOME NEW SPECIES FROM BRITISH COLUMBIA<sup>1</sup>

BY H. S. JACKSON AND ELIZABETH RUTH DEARDEN<sup>2</sup>

#### Abstract

In the present contribution four species of *Peniophora* and five of *Corticium*, collected in British Columbia and presumed to be new to science, are described and illustrated.

#### Introduction

In connection with a general study of the Thelephoraceae of Canada, the writers have had the privilege of studying a considerable number of collections made in British Columbia mostly by members of the staff of the Dominion Laboratory of Forest Pathology with headquarters at Victoria, B.C. During June and July 1948 the senior author spent several weeks collecting on Vancouver Island, during which period over six hundred specimens were obtained. Among the collections so far studied a number of species have been encountered that are apparently new to science. In the following pages nine such species, four in *Peniophora* and five in *Corticium*, are described and illustrated. Comments on the various species with suggestions as to relationships are furnished following the descriptions.

#### Description of Species

##### *Peniophora resinosa* sp. nov. (Fig. 1)

Fructificatio late effusa, ochraceo-fulva, adnata, 400–550  $\mu$  crassa; subiculum ex intertextis et partim paralleliter currentibus hyphis compositum, 3.5–4.5  $\mu$  latum, tunicis incrassatis, supra quas sunt hyphae tunicis tenuibus praeditae, nodoso-septatum; cystidia gracilia, clavata, 70–150  $\times$  7.5–12  $\mu$ , e subhymenio emergentia et 3/4 longitudinem extrusa, resinosa re supra incrassata, apice obtuso, tunicis irregulariter infra incrassatis; basidia clavata, 30–40  $\times$  6.5–7.5  $\mu$ , 4 sterigmata gerentia; basidiosporae late ellipsoideae, 7–9  $\times$  4.5–5.5  $\mu$ , tunicis tenuibus, levibus, non amyloideis.

Fructification widely effused on decorticated wood, cream buff, becoming darker with age, adnate, in section 400–550  $\mu$  thick, ceraceous, surface minutely porous to hispid due to emergent cystidia, sometimes becoming cracked in

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<sup>2</sup> Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out with the assistance of a grant in aid of research furnished by the University of Toronto.

Nos. I and II of this series appeared in *Can. J. Research, C*, 26 : 128–139 and 143–147. 1948.

<sup>3</sup> The writers are indebted to Miss Margaret H. Thomson for the preparation of the Latin diagnoses and to all those who have contributed specimens.

[The June issue of Section C (*Can. J. Research, C*, 27 : 45–145. 1949.) was issued July 14, 1949.]

age, margin undifferentiated, determinate, thinning out; subiculum with a basal layer of interwoven, in part horizontal, hyphae,  $3.5\text{--}4.5\ \mu$  broad with thickened walls, upper layer of more or less erect hyphae with thin walls, all with clamps, a considerable amount of resinous material present; cystidia numerous, slender, clavate,  $70\text{--}150 \times 7.5\text{--}12\ \mu$ , arising in the subhymenium and extruded  $3/4$  their length, incrustated above with resinous material, apex obtuse, walls thin above, irregularly thickened below, often with capillary lumen; basidia clavate  $30\text{--}40 \times 6.5\text{--}7.5\ \mu$ , bearing four subulate, slightly arcuate sterigmata; basidiospores broadly ellipsoid,  $7\text{--}9 \times 4.5\text{--}5.5\ \mu$ , flattened and appearing straight on one side with lateral apiculus, walls thin, smooth, nonamyloid.

Specimens examined:

**British Columbia:** On *Picea sitchensis*, Queen Charlotte City, Q.C. Isls. Aug. 19, 1944, R. E. Foster, OTB 16063 (V1711),\* OTB 16030 (V1714), OTB 16025 (V1723), OTB 16026 (V1732) **type**, OTB 16067 (V1736), OTB 16068 (V1929); on *Pseudotsuga taxifolia*, Cowichan Lake, Vancouver Isl., Aug. 1947, OTB 17618 (V2736).

Superficially this species somewhat resembles *P. gilvidula* Bres., based on material collected by J. R. Weir in Montana, but the cystidia and spores of *P. resinosa* are appreciably larger and the former species lacks clamps at the septa. Another species showing superficial resemblance is *P. Weirii* Bres., recorded only from Idaho. That species, however, is described by Burt as having gloecystidia as well as cystidia, the latter with thin walls. The spores also are much narrower than in *P. resinosa*, being described as cylindrical,  $6\text{--}8 \times 3\text{--}3.5\ \mu$ .

**Peniophora unica** sp. nov. (Fig. 2)

Fructificatio late effusa, ceracea, alba vel ochraceo-fulva,  $80\text{--}150\ \mu$  crassa, siccitate vernicosa membrana fit; subiculum e duabus straturis compositum, inferiore stratura e laxo intertextis hyphis altera  $2\text{--}3\ \mu$  diam., superiore e confertis rectis hyphis composita, nodoso-septatum; cystidia late obclavata vel subcylindracea,  $55\text{--}75 \times 12\text{--}16\ \mu$ , apice obtuso deinde maturitate crasse tunicato; basidia clavata vel cylindracea,  $18\text{--}20 \times 3\text{--}4\ \mu$ , 4 paene recta sterigmata gerentia; basidiosporae ellipsoideae,  $3\text{--}3.5 \times 2\text{--}2.5\ \mu$ , laterilater compressae, tunicis tenuibus, levibus, non amyloideis.

Fructification widely effused, ceraceous, white to cream buff, thin,  $80\text{--}150\ \mu$ , adnate, drying to a vernicose film, surface pruinose or minutely hispid due to projecting cystidia; subiculum of two layers of about equal thickness, the lower layer made up of loosely interwoven, nodulose hyphae,  $2\text{--}3\ \mu$  in diameter, the upper layer somewhat obscure but made up of closely compacted upright hyphae, all hyphae with clamps; cystidia broadly obclavate or subcylindrical, rarely fusiform,  $55\text{--}75 \times 12\text{--}16\ \mu$ , tapering to a narrow obtuse apex, when young thin walled and often with slight incrustations at apex, becoming thick walled and devoid of contents, often with one to four pseudosepta at maturity,

\* The herbarium numbers preceded by the letter V are the numbers of the herbarium of the Dominion Laboratory of Forest Pathology at Victoria, B.C. Those preceded by the letters OTB are in the mycological herbarium of the Division of Botany and Plant Pathology, Department of Agriculture, Science Service, Ottawa. All collections cited are also deposited in the mycological herbarium of the University of Toronto, TRT.

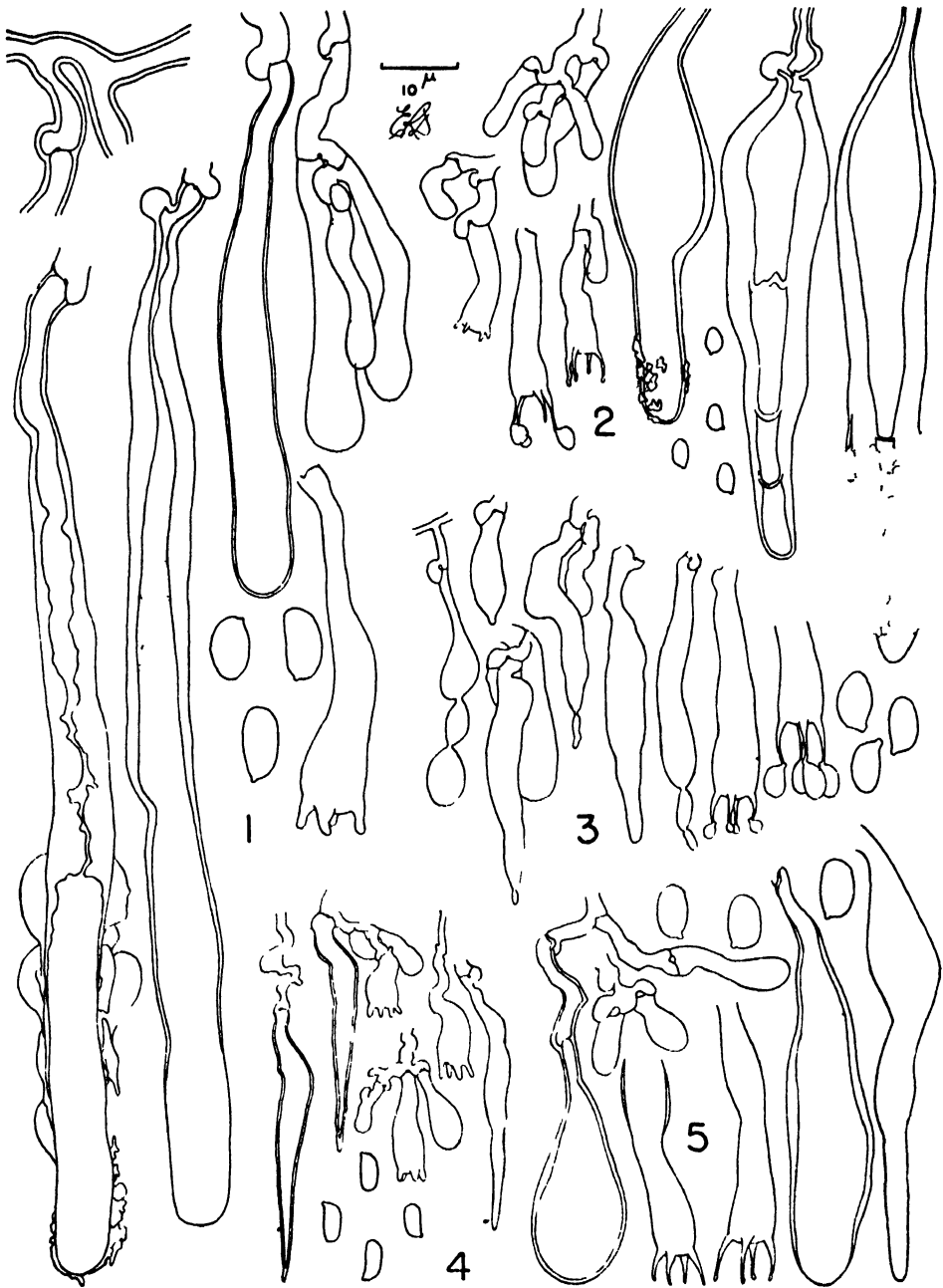


FIG. 1. *Peniophora resinosa*, FIG. 2. *Peniophora unica*, FIG. 3. *Peniophora inusitata*; FIG. 4. *Peniophora phlebioides*, all showing cystidia, basidia, and spores, FIG. 5. *Corticium testatum*, showing gloecystidia, basidia, and spores. Drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide - phloxine mounts, and reproduced at a magnification of approximately 1000 X.

embedded in the upper layer of the substratum or extending  $1/2-1/3$  length above hymenium; basidia clavate to cylindrical,  $18-20 \times 3-4 \mu$ , bearing four slender, nearly straight sterigmata; basidiospores ellipsoid,  $3-3.5 \times 2-2.5 \mu$ , laterally flattened with minute apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

**British Columbia:** On wood of *Abies lasiocarpa*, Prince George, P. J. Salisbury, Aug. 27, 1945, OTB 21416 (V4061) **type**.

The combination of the ceraceous character of the fructification, together with the large cystidia and minute spores, make this species unique in our experience. Its relationship with other described species is not clear.

***Peniophora inusitata* sp. nov. (Fig. 3)**

Fructificatio resupinata, late effusa, cremeo-ochracea, in superficie membranacea vel pelliculosa, separabilis; subiculum ex hyphis inferioribus in longitudinem, superioribus plus minusve recte dispositis compositum, nodoso-septatis; cystidia  $20-45 \times 4-5.5 \mu$ , subulata, leviter emergentia, apice moniliformi vel elongatis segmentis praedito; basidia clavata,  $20-25 \times 5.5-6.5 \mu$ , quattuor sterigmata gerentia; basidiosporae ellipsoideae vel obovatae,  $5.5-8 \times 3.5-5 \mu$ , tunicis levibus, non amyloideis.

Fructification resupinate, widely effused, separable, hymenial layer cream buff becoming pinkish cinnamon buff, membranous to pelliculose, occasionally rimose exposing the white fibrous lower subiculum, which is made up of longitudinally disposed hyphae of varying width  $1.5-3.5-5 \mu$ , with clamps and sometimes slightly enlarged at septa; subhymenial layer of upright, somewhat nodulose hyphae; cystidia  $20-45 \times 4-5.5 \mu$  arising from the same level as the basidia or slightly below, projecting about one-third their length, subulate, sometimes flexuous, frequently terminated by one or more moniliform swellings or tipped by one or more narrow elongated segments, which may be shed at maturity; basidia clavate, tapering to a narrow base, somewhat flexuous below, often subcylindric above,  $20-25 \times 5.5-6.5 \mu$ , bearing four slender sterigmata  $3.5-5 \mu$  long; basidiospores ellipsoid or asymmetrically obovate, rounded on either side with prominent lateral apiculus,  $5.5-8 \times 3.5-5 \mu$ , walls smooth, nonamyloid.

Specimen examined:

**British Columbia:** On wood of branch of *Populus trichocarpa*, Cinema, B.C., W. G. Ziller, Sept. 16, 1948, OTB 21417 (V3721) **type**.

The cystidia in this species might properly be interpreted as cystidioles and perhaps are in the nature of modified basidia. The relationship of the species with others is not at all clear.

***Peniophora phlebioides* sp. nov. (Fig. 4)**

Fructificatio effusa, adnata vel  $150 \mu$  crassa, alba vel cremea, molliter gelatinea, siccitate dein nonnihil crassa vernicosa stratura; subiculum obscurum, hyphis tunicis gelatineis praeditis, nodoso-septatum; cystidia acicularia,  $25-35 \times 3.5-4.5 \mu$ ,  $1/2-2/3$  longitudinem emergentia, apicem acuminatum versus angustiora, tunicis tenuibus vel leviter incrassatis, non incrustatis; basidia subcylindracea vel clavata,  $12-20 \times 3.5-4.5 \mu$ , 4 sterigmata gerentia; basidiosporae cylindraceae,  $4.5-5.5 \times 2-2.5 \mu$ , tunicis tenuibus, levibus, non amyloideis.

Fructification effused, adnate, up to 150  $\mu$  thick, white to cream becoming gray, when fresh soft gelatinous, drying to a somewhat thick vernicose layer, surface somewhat folded and with mucous strands, becoming cracked in age, margin thinning out; subiculum somewhat obscure, made up for the most part of hyphae with gelatinized walls, suberect in the lower part, in places with a more or less horizontal subhymenial layer looser in arrangement than in the lower subiculum; hyphae 1.5–2.5  $\mu$ , somewhat nodulose, walls gelatinized, with clamps; cystidia numerous, slender acicular, 25–35  $\times$  3.5–4.5  $\mu$ , arising from the subhymenium and emergent 1/2–2/3 their length, gradually narrowing to an acuminate apex, walls thin or slightly thickened, smooth, not incrustated; simple hyphalike paraphyses present in the hymenium; basidia subcylindrical to clavate, somewhat flexuous, 12–20  $\times$  3.5–4.5  $\mu$ , bearing four stout, upright sterigmata; basidiospores cylindrical, 4.5–5.5  $\times$  2–2.5  $\mu$ , flattened and appearing straight on one side, with lateral apiculus, walls thin, smooth, nonamyloid.

Specimens examined:

**British Columbia:** On *Pseudotsuga taxifolia*, Swartz Bay, North Saanich, Vancouver Isl., Sept. 23, 1943, I. Mounce, OTB 11523, 11546, **type**; New Denver, A. MacKinnon, July 3, 1947, OTB 17616 (V2721).

This ceraceous species appears to be amply distinct from others with which it might seem to be related. Bourdot & Galzin would probably have included it in *Corticium* in the group having tapering cystidioles where it would key to *C. subseriale* B. & G. The microscopic characters clearly show that it is neither that species nor the subspecies *C. ochraceo-fulvum* B. & G. Because of the tendency for folds in the hymenium the species approaches *Phlebia*. It is a matter of indifference at present whether such ceraceous species with small cystidia are included in *Corticium* or *Peniophora*. It seems probable that this group, part of which are now in *Corticium* and part in *Peniophora*, will sometime be combined in a separate genus with related forms now in *Phlebia*.

### ***Corticium testatum* sp. nov. (fig. 5)**

Fructificatio in elongatis vel fusiformibus areolis consistens, 3–8  $\times$  1–2 cm., ochraceo-fulva, ceracea, adnata, 50–75  $\mu$  crassa; subiculum ex intertextis hyphis gelatinosis tunicis praeditis compositum, nodoso-septatum; gloecystidia late clavata vel infra clavato-ventricosa et supra subcylindraceo-flexuosa, 30–55  $\times$  8–12  $\mu$ , tunicis tenuibus vel leviter incrassatis; basidia infra ventricosa, supra cylindracea, 30–40  $\times$  4.5–5  $\mu$ , maturitate supra hymenium extrusa, 4 leviter arcuata 5–6  $\mu$  longa sterigmata gerentia; basidiosporae late ellipsoideae, 6–7  $\times$  4–5  $\mu$ , tunicis tenuibus, levibus, non amyloideis.

Fructification forming irregularly elongated or fusiform patches 3–8  $\times$  1–2 cm., cream buff, ceraceous, adnate, 50–75  $\mu$  thick, margin thinning out abruptly; subiculum made up of fine interwoven hyphae with walls gelatinized, irregularly enlarged at intervals and these enlargements developing to cylindrical or pyriform structures with thick walls that appear irregularly scattered, developing in the hymenium to the more typical gloecystidia, clamps present throughout; gloecystidia variable, broadly clavate or clavate-ventricose below and subcylindrical-flexuous above, 30–55  $\times$  8–12  $\mu$ , walls thin or the clavate

ones sometimes with walls slightly thickened and indistinctly lamellate, contents guttulate becoming resinous; basidia few in the specimen examined, ventricose below, cylindrical above,  $30-40 \times 4.5-5.5 \mu$ , extending above the level of the hymenium about one-half their length at maturity, bearing four slightly arcuate, subulate sterigmata  $5-6 \mu$  long, walls sometimes slightly thickened below; basidiospores broadly ellipsoid,  $6-7 \times 4-5 \mu$ , somewhat flattened and appearing nearly straight on one side with lateral apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

**British Columbia:** On *Pseudotsuga taxifolia*, Cowichan Lake, Vancouver Isl., Mar. 14, 1947, P. J. Salisbury, OTB 21418 (V2627) **type**.

The broad gloecystidia, the walls of which become thickened below, are characteristic and might be interpreted as cystidia. The ventricose basidia, cylindrical above are also characteristic. The species seems amply distinct from other ceraceous gloecystidiolate forms that have been described.

### **Corticium praeteritum** sp. nov. (Fig. 6)

Fructificatio in parvis areolis effusa, 100–200  $\mu$  crassa, alba, adhaerens, subiculum hyphis intricatis tenuiter tunicatis, 1–3  $\mu$  diam., nodoso-septatis; gloecystidia cylindracea,  $30-60 \times 4-6 \mu$ , e subhymenio emergentia; aliae structurae quae in hymenio sunt, subpyriformes, vel abrupte globosae, capitatae,  $13-20 \times 6.5-9 \mu$ ; basidia gracilia  $25-30 \times 5-8 \mu$ , leviter ventricosa, 4 gracilia sterigmata gerentia; basidiosporae cylindraceae vel subellipsoideae,  $5-7.5 \times 2.5-3.5 \mu$ , tunicis tenuibus, levibus, non amyloideis.

Fructification effused in small areas, submembranous, dry, white, rather thin, 100–200  $\mu$ , adherent, margin thinning out, subiculum made up of closely interwoven hyphae, no horizontal basal layer; hyphae for the most part with thin walls, 1–3  $\mu$  in diameter, not gelatinized, a few are present with thickened walls and others intermixed with fine incrustation, clamps regularly present; gloecystidia long cylindrical or flexuous cylindrical, arising in the subhymenium,  $30-60 \times 4-6 \mu$ , sometimes tapering gradually from below, obtuse at apex, not projecting; other structures (modified basidia?) subpyriform or abruptly globose capitate arising in the hymenium,  $13-20 \times 6.5-9 \mu$ ; basidia slender,  $25-30 \times 5-8 \mu$ , slightly ventricose above the middle, bearing four slender, slightly arcuate sterigmata,  $3.5-4.5 \mu$  long; basidiospores cylindrical or subellipsoid,  $5-7.5 \times 2.5-3.5 \mu$ , flattened and appearing straight on one side with prominent lateral apiculus, walls thin, smooth, nonamyloid.

Specimen examined:

**British Columbia:** On wood of *Alnus rubra*, Mt. Douglas Park, Vancouver Isl., H. S. Jackson, July 2, 1948, **type** in TRT.

The presence of the subpyriform or globose capitate structures, which may be modified basidia, in the hymenium together with the occurrence of typical gloecystidia makes this species unique in our experience. The relationship is not clear but obviously not with the "Gloeocystidiales" section of *Peniophora* in the Bourdot & Galzin classification.

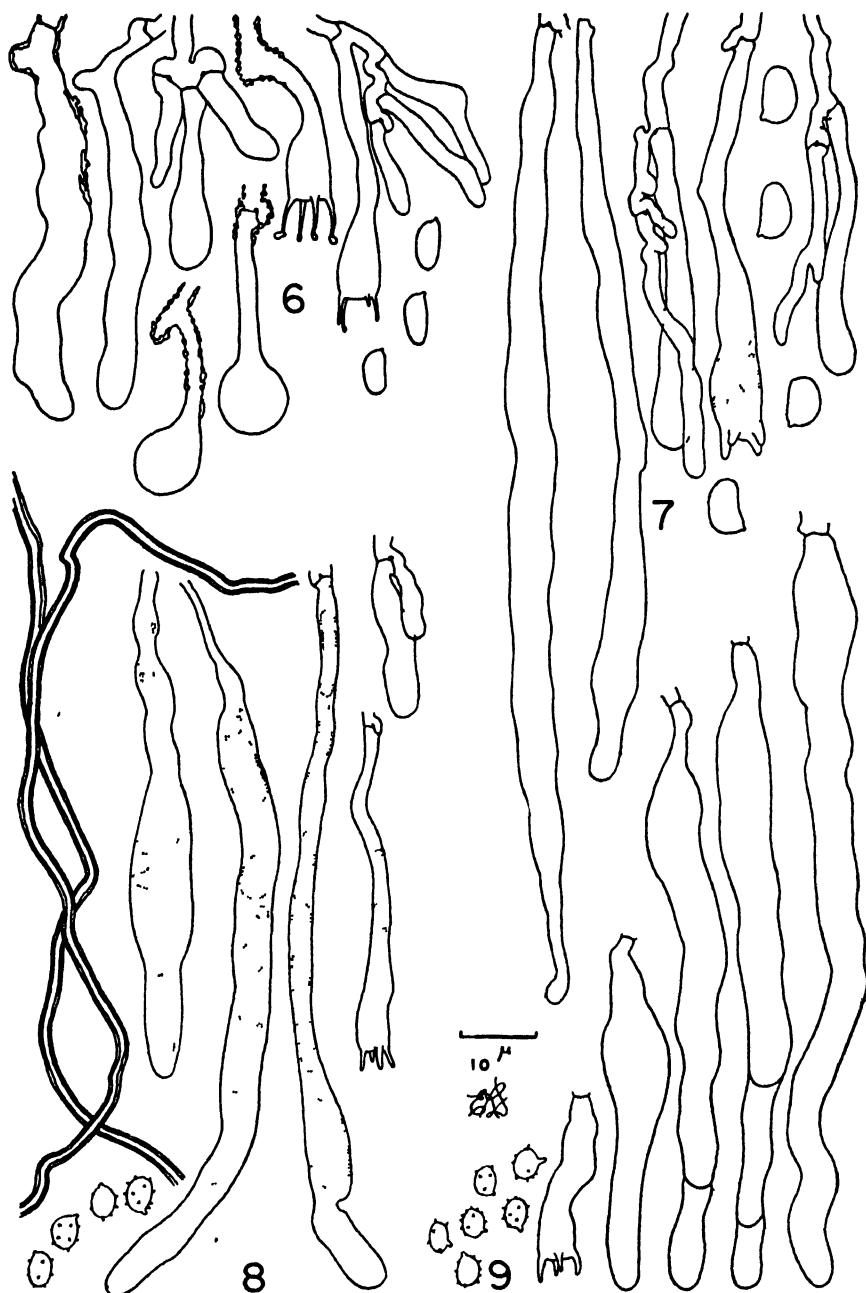


FIG. 6. *Corticium praeteritum*; FIG. 7. *Corticium separatum*; FIG. 8. *Corticium quaesitum*; FIG. 9. *Corticium propinquum*, all showing gloecystidia, basidia, and spores. In Fig. 6 below, are two globose capitate structures from hymenium, and in Fig. 8, to the left, subicular fibrous hyphae. Drawn to a uniform scale with the aid of a camera lucida from potassium hydroxide - phloxine mounts, and reproduced at a magnification of approximately 1000 $\times$ .



**Corticium separatum** sp. nov. (Fig. 7)

Fructificatio late effusa, ceracea, ochracea, adnata, 600–900  $\mu$  crassa; subiculum ex erectis nodulosus hyphis compositum, 2–3  $\mu$  diam., nodoso-septatum; gloeocystidia cylindracea vel anguste fusiformia, ad apicem obtusa, 65–130  $\times$  4.5–6  $\mu$ ; basidia graciliter clavata, 45–50  $\times$  5.5–6.5  $\mu$ , 4 gracilia subulata 5–5.5  $\mu$  longa sterigmata gerentia; basidiosporae oblongae cylindraceae, 7–8  $\times$  3.5–4.5  $\mu$ , tunicis tenuibus, levibus, non amyloideis.

Fructification ceraceous, becoming indurated and rimose, ochraceous, widely effused, adnate at first but separable when dry, 600–900  $\mu$  thick, margin thinning out to a vernicose film; subiculum made up of upright nodulose hyphae 2–3  $\mu$  in diameter, often collapsed, with clamps; gloeocystidia long, cylindrical or narrowly fusiform, subflexuous, 65–130  $\times$  4.5–6  $\mu$ , obtuse at apex, arising at different levels in the subiculum and extending to surface of hymenium, not projecting; basidia slenderly clavate, 45–50  $\times$  5.5–6.5  $\mu$ , bearing four subulate, slender sterigmata, 5–5.5  $\mu$  long; basidiospores oblong cylindrical, 7–8  $\times$  3.5–4.5  $\mu$ , flattened and slightly concave on one side, walls thin, smooth, nonamyloid.

Specimen examined:

**British Columbia:** On *Abies grandis*, Cadboro Bay, Vancouver Isl., Nov. 21, 1940, J. E. Bier, OTB F10111 type.

There are not many truly ceraceous species of *Corticium* having gloeocystidia and all such with which we are familiar except *C. testatum* described above and *C. umbratum* B. & G. have spore walls showing some degree of amyloidity. *C. umbratum*, according to Lundell who transferred the species to *Gloeocystidium*, has gloeocystidia and the spores in available material are not at all amyloid. The species described above differs markedly from *C. umbratum* in gross appearance and in the measurements of both gloeocystidia and spores. The former also has regular clamps at the septa which are absent or rare in the latter.

**Corticium quaesitum** sp. nov. (Fig. 8)

Fructificatio late effusa, 400–600  $\mu$  crassa, margine abrupto, floccosa; subiculum partim e dense intertexta basali stratura fibratarum, crasse tunicatarum, 1.5–2.5  $\mu$  diam., hypharum quae in solutione Melzer fuscae fiunt compositum, tenue tunicatis hyphis gloeocystidia et basidia supra producentibus; gloeocystidia cylindracea vel clavata, 60–120  $\times$  5.5–8  $\mu$ ; basidia gracilia clavata, 35–45  $\times$  5–5.5  $\mu$ , 4 sterigmata gerentia; basidiosporae subglobosae vellate vel ellipsoideae, 3.5–4.5  $\times$  3–3.5  $\mu$ , tunicis tenuibus, minute asperulatis, fortiter amyloideis.

Fructification widely effused, cream buff, 400–600  $\mu$  in thickness, surface subceraceous, broken into small tufted areolae, exposing the woolly floccose subiculum, margin abrupt floccose; subiculum made up in part of densely interwoven basal layer of fibrous nonseptate hyphae, 1.5–2.5  $\mu$  in diameter, with thick walls, turning brown in Melzer's solution; an upper layer of more or less upright thin-walled hyphae with clamps gives rise to the gloeocystidia and basidia; gloeocystidia numerous, cylindrical to clavate, somewhat flexuous, 60–120  $\times$  5.5–8  $\mu$ , contents guttulate becoming pseudoseptate after withdrawal of contents; basidia slenderly clavate, 35–45  $\times$  5–5.5  $\mu$ , bearing four

upright, subulate sterigmata; basidiospores subglobose or broadly ellipsoid,  $3.5\text{--}4.5 \times 3\text{--}3.5 \mu$ ; slightly depressed on one side with inconspicuous apiculus, walls thin, minutely asperulate, strongly amyloid.

Specimen examined:

**British Columbia:** On decayed log of *Pseudotsuga taxifolia*, Nitinat River, Vancouver Isl., Oct. 30, 1946, P. J. Salisbury, OTB 17109 (V2596) type.

The only collection available is not in the best of condition and the above description of the fructification may need emendment when other material becomes available. The species however possesses such an unusual combination of characters that it seems desirable that it be placed on record. It may be distinguished from all others known to us by the presence in the subiculum of fiber hyphae with pseudoamyloid walls, together with the long slender gloecystidia and the strongly amyloid spores with minutely roughened walls.

The presence of the pseudoamyloid hyphae in the subiculum serves to relate the species to *Corticium odoratum* (Fr.) B. & G., *C. galactinum* Fr., *C. abeuns* Burt (= *Gloeocystidium ochroleucum* Bres. & Torr.), and *C. praestans* Jackson. As has been previously noted by the writer, this character is suggestive of a possible relationship with *Vararia* (= *Asterostromella*). The rough amyloid spores taken together with the pseudoamyloid hyphae suggest a possible relationship for the species here described to the group of *Vararia* species having similar rough-walled amyloid spores such as *Vararia granulosa* (Pers. ex Fr.) Laurila.

### ***Corticium propinquum* sp. nov. (Fig. 9)**

Fructificatio late effusa, vel in areolis, cremeo-alba, adnata  $125\text{--}200 \mu$  crassa, margine abrupto; subiculum ex lateis hyphis compositum, non nodoso-septatum; gloecystidia flexuosa, cylindracea vel subclavata,  $50\text{--}90 \times 6\text{--}8.5 \mu$ , e basi subiculi emergentia; basidia cylindracea,  $20\text{--}25 \times 4.5\text{--}5.5 \mu$ , infra ventricosa, 4 paene recta  $4 \mu$  longa sterigmata gerentia; basidiosporae subglobosae,  $3.5\text{--}4 \times 3\text{--}3.5 \mu$ , apiculo prominente, tunicis tenuibus, verrucosis, fortiter amyloideis.

Fructification broadly effused, or in small irregular areas, creamy white, adnate,  $125\text{--}200 \mu$  thick, surface soft membranous, becoming rimose, exposing the substratum, margin abrupt; subiculum with a few horizontal hyphae but for the most part with upright, thin-walled, mostly collapsed hyphae without clamps; gloecystidia numerous, flexuous, cylindrical or subclavate, often ventricose below,  $50\text{--}90 \times 6\text{--}8.5 \mu$ , arising from the base of the subiculum or at various levels and extending to the surface of the hymenium, not noticeably projecting; basidia cylindrical,  $20\text{--}25 \times 4.5\text{--}5.5 \mu$ , ventricose or subventricose below, bearing four nearly straight or slightly arcuate sterigmata  $4 \mu$  long; basidiospores subglobose,  $3.5\text{--}4 \times 3\text{--}3.5 \mu$ , with prominent peglike apiculus, walls thin, verrucose, strongly amyloid.

Specimen examined:

**British Columbia:** On log of *Thuja plicata*, Cowichan Forest Experiment Station, Lake Cowichan, B.C., Sept. 21, 1943, R. E. Foster, OTB 21419 (V379) **type**.

The relationship of this species is probably not with *C. quaesitum* described above but with such gloeocystidiate forms having amyloid spore walls as *Corticium porpsum* B. & C. and *Gloeocystidium Karstenii* B. & G. It differs from the former in the absence of clamps and from the latter in the smaller rough-walled spores, in gross characters of the fructification, and occurrence on a coniferous substratum.

## THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

### I. THE STATUS OF THE ENDOGENOUS RESPIRATION<sup>1</sup>

BY FLORA C. NORRIS,<sup>2</sup> JACK J. R. CAMPBELL,<sup>3</sup> AND PHYLLIS W. NEY<sup>4</sup>

#### Abstract

The endogenous respiration of *Pseudomonas aeruginosa* was shown to differ in several respects from that of other organisms. Although values obtained for the degree of oxidative assimilation of glucose agree with those reported by other workers, this synthesis of storage products was found not to be inhibited by the usual concentrations of 2,4-dinitrophenol. The techniques commonly employed to reduce stored products had no effect on this organism. The endogenous respiration was shown to function normally in the presence of added substrate.

In studies of aerobic microorganisms the influence of the rate of endogenous respiration is of importance in interpreting data relative to metabolism. However, little attention has been paid to the problem of the endogenous respiration of these microorganisms.

The most common approach to the problem of endogenous respiration is to work with cultures whose endogenous activity has been reduced to a point where it is not considered a serious interfering factor. This is usually done by devising a medium that produces cells with good metabolic activity but with practically no storage materials (14). It is not always possible to produce cells with low storage materials, and other means of obtaining cells with reduced endogenous activity, such as aeration of the resting cell suspension or aging at low temperatures, have been employed. Quastel and Whetham, (10) working with *Escherichia coli*, were the first to suggest the starvation technique now commonly used. This technique consists of vigorously aerating a resting cell suspension in non-nutrient solution for two to four hours before use, thus forcing the organisms to oxidize their stored products.

Many workers report that endogenous respiration is suppressed in the presence of a readily oxidizable substrate and thus should be disregarded. Stier and Stannard (12) working with baker's yeast, Clifton (5) with *Pseudomonas calco-acetica*, Clifton and Logan (6) with *E. coli*, Doudoroff (7) with *P. saccharophila*, Bernstein (2) with *P. saccharophila*, and Barker (1) with *Protoheca zopfii* concluded that endogenous respiration is completely inhibited by substrate. On the other hand Reiner, Gest, and Kamen (11), by the use of radioactive carbon, obtained data indicating a stimulation of the endogenous respiration of yeast in the presence of glucose or acetate.

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Contribution from the Department of Dairying, The University of British Columbia, Vancouver, B.C. This study was carried out under a grant from the National Research Council of Canada, laboratory facilities being found by The University of British Columbia.

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The present study was undertaken to clarify the status of the endogenous respiration of the obligate aerobe, *P. aeruginosa*, particularly with reference to the influence of oxidizable substrate. This information is an essential prerequisite to a detailed study of the metabolism of this organism.

### Methods

The culture of *P. aeruginosa* A.T.C. 9027, used throughout, was a typical, strongly pigmenting strain. A study of the nutrition of the organism as related to pigment production has previously been reported (3, 4, 8).

Stock cultures of the organism were maintained in a liver extract gelatin agar under refrigeration conditions after initiation of growth at 30° C. For use in experimental work the culture was transferred two to three times at 24 hr. intervals in Sullivan's medium (13). In order to maintain a vigorous undissociated culture, a fresh transfer was taken semimonthly from a refrigerated stock. The medium used in growing cells for Warburg experiments consisted of 0.3% ammonium dihydrogen phosphate, 0.1% magnesium sulphate septahydrate, 0.3% carbon source, and 0.5 p.p.m. of iron added as ferric chloride. The medium was brought to neutrality and dispensed in 100 ml. quantities in Roux flasks. After sterilization a 10% solution of dipotassium hydrogen phosphate was added aseptically to a final concentration of 0.3%. The inoculum used was a 24 hr. culture in Sullivan's medium. The cells were harvested after incubation for 18 to 24 hr. at 30° C., washed with half the growth volume of 0.9% saline, and finally made up to the desired concentration. The concentration of cells in the washed suspension, as determined turbidimetrically by a Fisher Electrophotometer, was measured before each experiment.

A conventional Warburg apparatus was used to follow the oxygen uptake and carbon dioxide production of the cell suspensions. In studies of the influence of inhibiting substances on oxygen uptake the cells were incubated 20 min. with the inhibitor prior to the addition of substrate from the sidearm. All Warburg experiments were run at 31° C. The reaction mixture in the cups was pH 7.2.

Cells subjected to the aeration technique were prepared as described above except that aseptic technique was observed throughout. The concentrated cells were aerated vigorously at room temperature while cells that had remained undisturbed at room temperature served as a control. At specified intervals samples of aerated and control cells were removed and their respective respiratory rates determined.

### Experimental

#### *Nature of Storage Products*

In order to determine the nature of the storage products of *P. aeruginosa*, respiration quotient values were obtained using heavy suspensions of cells that had been grown with glucose, ammonium succinate, or sodium acetate as the sole source of carbon. Glucose and succinate cells were harvested at

24 hr. and brought to 20 times growth concentrations while acetate cells were harvested at 24 and 49 hr. and brought to 40 times growth concentration. Cell suspensions of essentially the same turbidity were obtained in all cases. From Table I it can be seen that identical R. Q. values were obtained in all cases indicating that the storage product of the cells is the same regardless of growth substrate or age and is a compound that is slightly oxidized.

TABLE I  
R.Q. OF CELL SUSPENSIONS IN THE ABSENCE OF SUBSTRATE

Growth substrate	Age of cells, hr.	R.Q.
Glucose	24	1.11
Succinate	24	1.12
Acetate	24	1.13
Acetate	49	1.11

#### *Influence of Substrate*

In order to determine the influence of concentration of substrate on the endogenous respiration, cells were suspended at 20 times growth concentration and allowed to oxidize 2, 3, 4, and 5  $\mu M$  of glucose for 70 min. in the Warburg apparatus. These concentrations require 12, 18, 24, and 30  $\mu M$  oxygen respectively for complete oxidation. A similar experiment was set up using 6, 9, 12, and 15  $\mu M$  of acetate (equivalent to 12, 18, 24, and 30  $\mu M$  oxygen). If the endogenous respiration is disregarded there is found to be a variation of 40% in the degree of oxidation of a given substrate depending upon its concentration and, with the lower concentrations of substrate, the oxygen uptake is shown to be greater than is theoretically possible. If, on the other hand, the endogenous respiration is considered to function normally in the presence of substrate, and the oxygen uptake due to the substrate dissimilation is determined by subtracting the endogenous oxygen uptake from the total uptake, the degree of oxidation is found to be comparable for all substrate concentrations. Moreover, the extent of oxidation is in agreement with the data reported in the literature for related organisms. According to the concept of oxidative assimilation proposed by Barker (1) and Clifton (5), microorganisms oxidize a portion of the energy source to carbon dioxide and water and assimilate the remainder directly into cellular material. The figures usually reported are approximately two-thirds oxidation and one-third assimilation, which is the ratio obtained with *P. aeruginosa* if oxygen uptake due to endogenous respiration is subtracted from the total (Table II).

In order to determine the influence of concentration of cells on amount of oxygen taken up in the presence of a constant amount of substrate, cells harvested from the glucose medium were resuspended at 10, 20, and 40 times growth concentration. The activity of these cell suspensions was determined in the Warburg respirometer with 5  $\mu M$  of glucose as substrate. If

TABLE II

OXIDATIONS OF GLUCOSE-GROWN CELLS ON VARYING CONCENTRATIONS OF GLUCOSE AND ACETATE

Warburg substrate	Conc. in cup, $\mu M$	Total O <sub>2</sub> uptake, $\mu l.$	Theoretical uptake, $\mu l.$	Percentage of theoretical	O <sub>2</sub> uptake (endogenous subtracted), $\mu l.$	Percentage of theoretical (endogenous subtracted)
Glucose	Nil	152				
	2*	336	270	124	184	68
	3	425	402	106	273	68
	4	505	535	92	353	66
	5	565	672	84	413	61.5
Acetate	Nil	153				
	6**	354	270	131	201	74.5
	9	453	402	113	300	75
	12	530	535	99	377	70.5
	15	625	672	93	470	70

\* 22 hr. cells.

\*\* 26 hr. cells.

the endogenous respiration has been repressed, the same total oxygen uptake should be obtained in all cases since the same amount of substrate is being oxidized by each of the above cell suspensions.

The results recorded in Table III show, however, that the total oxygen uptake increases proportionately with an increase of endogenous respiration. If, on the other hand, the endogenous respiration is subtracted, the percentage

TABLE III

OXIDATION OF 5  $\mu M$  GLUCOSE BY CELL SUSPENSIONS OF VARYING CONCENTRATION

Concentration of cells	O <sub>2</sub> uptake, endogenous, $\mu l.$	O <sub>2</sub> uptake 5 $\mu M$ Glucose		
		Total, $\mu l.$	Minus endogenous, $\mu l.$	Percentage of theoretical uptake
10×	37	420	383	57
20×	95	540	445	66
40×	200	645	445	66

oxidation is found to be relatively constant in all cases (Table III). The lower value obtained with cells concentrated 10 times is explained by the fact that the curve for these cells had not leveled off at 80 min.

The data in Tables II and III show conclusively that the endogenous respiration of *P. aeruginosa* is unaffected by the presence of oxidizable substrate. It follows that in determining the oxygen uptake on a given substrate, the endogenous respiration must be subtracted from the total uptake.

### *Influence of Aeration and Storage*

In studies on the influence of aeration and storage on endogenous respiration, 24 hr. glucose-grown cells were resuspended in saline at 10 times growth concentration and the suspension divided into two portions. One portion of the suspension was aerated vigorously at room temperature while the other half, serving as control, was allowed to stand undisturbed. Samples of aerated cells were removed at intervals of two, five, and eight and one-half hours while samples of control cells were removed at zero, two, five, and eight and one-half hours. The endogenous activity and the ability to oxidize glucose were determined. The purity of suspensions was checked by Gram stain at the conclusion of the experiment.

Since results similar to those obtained after aeration for eight and one-half hours were also obtained at two and five hours, curves depicting the results for only zero and eight and one-half hours are recorded in Fig. 1. From these

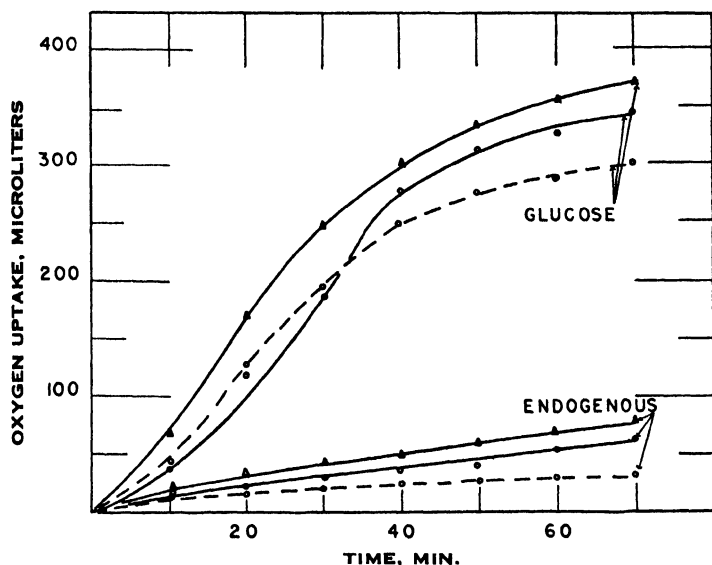


FIG. 1. The effect of aeration on resting cells of *P. aeruginosa*. Warburg cups contained 0.5 ml. of cell suspension, 1.5 ml. of *M/15* phosphate buffer, and 0.2 ml. of substrate. Final volume was 3.0 ml.

- △ ———— △ = Un-aerated cells, zero hours.
- ———— ○ = Aerated cells, eight and one-half hours.
- - - - - - ○ = Control cells, eight and one-half hours.

data it can be seen that there is no significant decrease in the endogenous respiration nor is there any appreciable decrease in the ability of the cells to oxidize glucose after aeration for eight and one-half hours. The slight decrease in activity at eight and one-half hours cannot be attributed to aeration since control cells showed a similar slight decrease. The use of aeration is a recognized procedure for reducing the endogenous respiration



of microorganisms. The inability of this technique to affect the autorespiration of *P. aeruginosa* indicates that there may be something unique about the storage products of the organism.

Additional data on the influence of storage conditions were obtained when a cell suspension was stored in the refrigerator and samples removed daily for study. The endogenous respiration and ability to oxidize glucose were determined. Results recorded in Table IV show that after four days' storage there was no change in the activity of the cells.

TABLE IV  
STORAGE OF CELLS

Time of storage, days	Oxygen uptake at 70 min.	
	Endogenous, $\mu$ l.	3 $\mu$ M glucose, $\mu$ l.
0	60	290
1	53	310
2	57	294
3	53	312
4	57	285

### *Inhibition of Synthesis*

Since *P. aeruginosa* appears to be exceptional in its behavior when aerated and since it has been shown to possess a mechanism for the oxidative assimilation of substrates, it was considered of value to determine the action of compounds that inhibit synthetic processes.

Sodium azide and 2,4-dinitrophenol have been used extensively as inhibitors of oxidative assimilation (5, 6, 7). Solutions of sodium azide at final concentrations of  $M/200$ ,  $M/400$ , and  $M/1000$  and 2,4-dinitrophenol at  $M/1000$ ,  $M/1500$ ,  $M/2000$ ,  $M/2500$ ,  $M/4000$ , and  $M/8000$  were used in Warburg experiments with glucose as substrate. In the calculation of these results the respective endogenous respiration values were subtracted from those obtained in the presence of glucose. The presence of dinitrophenol produced no inhibition of oxidative assimilation in any of the concentrations used.  $M/1000$  azide, however, increased the oxidation from 64% to 90% of the theoretical value. Higher concentrations of azide inhibited both rate and total amount of oxygen uptake.

### Discussion

From this study it is evident that one cannot make generalizations regarding the endogenous respiration of microorganisms since the endogenous respiration of *P. aeruginosa* has been shown to differ in several important respects from that reported for other organisms. Although data obtained using azide as an

inhibitor of oxidative assimilation agree with those reported in the literature for other organisms, 2,4-dinitrophenol, the most common reagent employed for this purpose, failed to inhibit the oxidative assimilation of *P. aeruginosa*. It was also found that the endogenous respiration of this organism could not be reduced by either vigorous aeration or storage of the organism at low temperatures, indicating a possible peculiarity in the nature of the storage products elaborated or the destruction by this technique of certain enzymes or co-enzymes associated with the utilization of this storage product. The conclusion that the endogenous respiration functions normally in the presence of added substrate and thus should be subtracted in the calculation of the degree of oxidation of the substrate contradicts the practice followed by most workers.

It is possible that the type of endogenous respiration exhibited by *P. aeruginosa* is common to organisms that do not have a constitutive enzyme system for oxidizing glucose (9). The lack of such enzymes indicates that the storage products are not oxidized via glucose or closely related compounds. The enzymes required for the oxidation of stored products thus would not compete with those involved in the oxidation of the substrate.

Much of the confusion and many of the contradictory results reported in the literature may be explained by the fact that there is considerable variation in the nature of the endogenous respiration of various microorganisms. The significance that is to be attached to the rate of endogenous respiration in interpreting data on the intermediary metabolism of microorganisms is thus shown to be of paramount importance.

### Acknowledgment

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## THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

### II. LIMITATIONS OF SIMULTANEOUS ADAPTATION AS APPLIED TO THE IDENTIFICATION OF ACETIC ACID, AN INTERMEDIATE IN GLUCOSE OXIDATION<sup>1</sup>

BY JACK J. R. CAMPBELL,<sup>2</sup> FLORA C. NORRIS,<sup>3</sup> AND MARGARET E. NORRIS<sup>4</sup>

#### Abstract

Acetic acid has been isolated as an intermediate compound in the oxidation of glucose or  $\alpha$ -ketoglutaric acid by *Pseudomonas aeruginosa*. Cells of this organism produced under conditions of intense aeration were found to have lost the ability to oxidize acetic acid and this compound was found in large quantities in the growth medium. When these cells were used to carry out the oxidation of glucose, it was found that the oxygen consumed was the amount needed to convert glucose to acetic acid, thus confirming the role of acetate as an intermediate in glucose oxidation. In contrast to the above-mentioned criteria the technique of simultaneous adaptation ruled out acetic acid as a possible intermediate in the oxidation of either glucose or  $\alpha$ -ketoglutaric acid. The status of the theory of simultaneous adaptation is discussed in relation to the data obtained.

Progress towards a fuller understanding of the anaerobic metabolism of bacteria has been very rapid during the past twenty years. In contrast little advancement has been made toward the elucidation of the path by way of which bacteria oxidize carbohydrates. Certain valuable contributions such as Lipmann's (4) analysis of the pyruvate oxidizing system of *Lactobacillus delbrueckii* and Barron's (1, 2) studies on the oxidations of the gonococci and streptococci have been made. However, the majority of such studies on oxidation have been concerned with a minor phase of the metabolism of a fermentative organism. Information concerning the carbohydrate metabolism of an obligate aerobe such as *Pseudomonas aeruginosa* is meager. Pervozvanskii (8), by the use of vigorous aeration, and high quantities of substrate in the presence of calcium carbonate, was able to block the aerobic metabolism of fluorescing organisms in the earlier phases and thereby cause large quantities of gluconic and ketogluconic acids to accumulate in the medium. The production of the latter compound under these vigorous conditions has also been demonstrated for *P. aeruginosa* A.T.C. 9027 (6). A further step in the breakdown of glucose was indicated when Lockwood and Stodola (5) showed that 16 to 17% yields of  $\alpha$ -ketoglutaric acid were obtained if the oxidation of 2-ketogluconic acid by *Pseudomonas fluorescens* was allowed to continue until the medium had lost all reducing power. Since the conditions to which the organism was subjected during these large scale fermentations were so far removed from those of its normal environment, it is impossible

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to conclude from these data that the acids produced are normal intermediates in glucose dissimilation. However, they are indicative of the mechanism available to the cell.

Since *P. aeruginosa* has been shown to have an adaptive system for the oxidation of glucose (6), it was thought that it would be a simple matter to eliminate or identify the compounds intermediate in the dissimilation of glucose by the use of the technique of simultaneous adaptation as proposed by Stanier (9). However, when cells were harvested from a growth medium in which glucose was the source of energy, and tested on postulated intermediate compounds, it was found that, with the exception of gluconic acid, a period of adaptation was required before the cell could oxidize any of the substrates. According to Stanier's interpretation, this would mean that of the 50 substances tested only gluconic acid could be an intermediate. Since this list included such compounds as 2-ketogluconic, pyruvic, and acetic acids, the criterion adopted for the elimination of these compounds as possible intermediates would appear to be questionable.

The object of the present investigation was to attempt to identify acetic acid as an intermediate in the oxidation of glucose by *P. aeruginosa* and to check on the soundness of the technique of simultaneous adaptation.

### Methods

The methods used in the present study are in a large measure the same as those recorded previously (7). *Pseudomonas aeruginosa* A.T.C. 9027 was used throughout. Any variations in the methods employed are detailed below. The medium used in growing cells for Warburg experiments consisted of 0.3% ammonium dihydrogen phosphate, 0.1% magnesium sulphate septahydrate, and 0.5 p.p.m. of iron added as ferric chloride. The medium was brought to neutrality before autoclaving. After sterilization, 0.3% of an autoclaved 10% solution of dipotassium hydrogen phosphate and glucose,  $\alpha$ -ketoglutarate or acetate that had been sterilized by passage through a sintered glass filter, were added aseptically.

In order to isolate a quantity of volatile acid adequate for identification purposes the following procedure was carried out for both glucose and  $\alpha$ -ketoglutarate. Two liters of media were prepared as previously described and one liter of this was held as the uninoculated control while the other was dispensed in Roux flasks in 100 ml. quantities. The 10 flasks were inoculated and then incubated at 30° C. for 18 hr. At the end of this time the cells were centrifuged down, the supernatant acidified with sulphuric acid, and two liters of distillate collected by steam distillation. The distillate was neutralized, concentrated on a hot plate to about 150 ml., reacidified with sulphuric acid, and about 130 ml. collected by slow distillation. The liter of uninoculated medium, which contains substrate and phosphate, was treated similarly with the exception that the cells from 100 ml. of the inoculated medium were added before distillation to ensure the presence of at least as much protein as was

present in the liter of supernatant obtained from the inoculated media. The distillates from these controls were alkaline to bromthymol blue; the procedure was thus not carried beyond the initial distillation. Duclaux constants were run on the distillates from the inoculated media. The procedure was as follows: 25 ml. of sample was set aside while exactly 100 ml. was placed in a 250 ml. distilling flask equipped in the usual manner for such determinations. After discarding the first 10 ml. of distillate the subsequent three 25 ml. fractions were collected. These three samples plus the original 25 ml. aliquot were then titrated using a Beckman potentiometer. Three end points were taken for each sample—pH 6.0, 7.5, and 8.5. Essentially the same constants were obtained at each of the end points. Titrations required from 3 to 12 ml. of *N*/400 sodium hydroxide. Standards of formic, acetic, and propionic acids of about the same concentrations as the unknowns were also run.

### Experimental

According to the theory of "simultaneous adaptation" cells harvested from a medium containing a specific energy source will attack this substrate or any of the compounds that are intermediate in its breakdown, and therefore present during growth, without a period of adaptation. If acetic acid is an intermediate in the oxidative breakdown of glucose by *P. aeruginosa*, cells of this organism harvested from a glucose medium and tested in a Warburg respirometer should immediately oxidize acetate at a maximum rate. From Fig. 1 it can be seen that this is not the case and therefore, on the basis of the theory of simultaneous adaptation, acetic acid would thus be ruled out as an intermediate in glucose oxidation by this microorganism. When  $\alpha$ -ketoglutaric acid was the sole energy source for growth, it was shown that resting cells harvested from this medium also went through a period of adaptation when placed in a Warburg cup with acetate as substrate. However cells that had been harvested from a medium in which acetate was the only source of carbon did oxidize this compound vigorously and immediately, Fig. 1.

In order to determine whether or not acetate or at least some volatile acid was formed during the oxidation of glucose or  $\alpha$ -ketoglutarate, cells were harvested from the liquid media containing one or other of these carbon sources and allowed to oxidize their parent substrate in 125 ml. Warburg cups. The reaction mixture, which had a total volume of 30 ml., contained 9 mgm. of substrate. The oxidations were stopped by the addition of sulphuric acid and the contents of the flask subjected to steam distillation. Titrations of the distillate with *N*/100 sodium hydroxide are shown in Table I. In Experiment V an atmosphere of 10% air and 90% nitrogen was used in the hope that intermediate compounds would accumulate because the limiting oxygen prevented their further oxidation. However, the results were similar to those of the four experiments run in an atmosphere of air. In every case, small amounts of volatile acid were formed during the oxidation of glucose or  $\alpha$ -ketoglutarate by resting cells of *P. aeruginosa*.

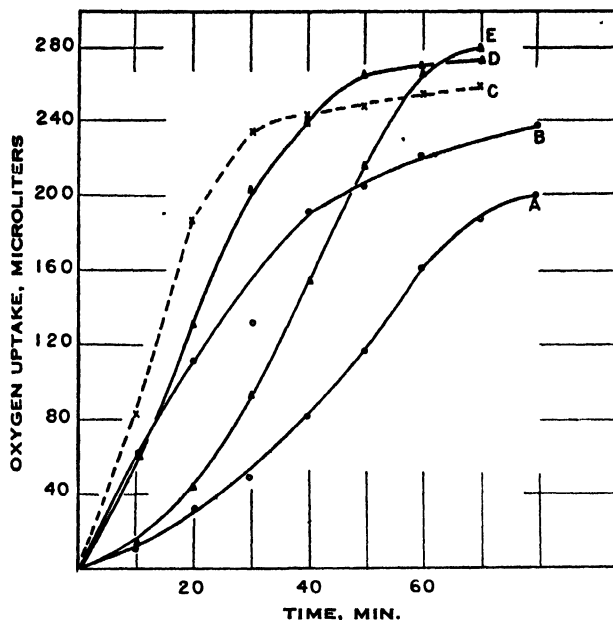


FIG. 1. Adaptation of glucose and  $\alpha$ -ketoglutarate-grown cells to the oxidation of acetate.

Warburg cups contained 0.5 ml. of cell suspension; 1.5 ml. of M/15 phosphate buffer, pH 7.2; 0.2 ml. of substrate. Final volume was 3.0 ml. Theoretical oxygen uptake for complete oxidation of any substrate was 403 microliters. Warburg substrates; A, C, and E, acetate; B,  $\alpha$ -ketoglutarate; D, glucose. Value of endogenous respiration has been subtracted.

○ — — — — — ○ =  $\alpha$ -Ketoglutarate-grown cells.  
 △ — — — — — △ = Glucose-grown cells.  
 × - - - - - × = Acetate-grown cells.

TABLE I

PRODUCTION OF VOLATILE ACID BY RESTING CELL SUSPENSIONS

Experiment	Substrate	Ml. of N/100 NaOH to neutralize distillate when reaction stopped at		
		10 min.	15 min.	60 min.
I	Endogenous ( $\alpha$ -ketoglutarate)	— 1.55	1.25 1.40	— —
II	Endogenous (glucose)	— 1.80	1.10 1.85	— —
III	Endogenous (glucose)	— —	1.30 1.85	— —
IV	Endogenous (glucose)	— —	1.25 2.95	— —
V	Endogenous (glucose)	— —	— —	1.25 1.50

In an effort to identify this volatile acid the supernatant from a liter of 18 hr. culture, in which glucose or  $\alpha$ -ketoglutarate had been the only source of carbon, was acidified and steam distilled. Duclaux constants were determined for the concentrated distillates and compared to the values obtained for comparable concentrations of formic, acetic, and propionic acids. Repeated determinations gave similar findings. The results for the distillates of the growth media are seen to parallel those obtained for acetic acid. The constants for acetate differ from those usually reported in the literature but apparently they are an idiosyncrasy of the apparatus or more probably of the dilute solutions used, since the constants usually given are for much more concentrated solutions. Under any circumstance this idiosyncrasy can be used as a criterion for showing definitely that the volatile acid formed during growth is almost solely acetic acid.

TABLE II

DUCLAUX CONSTANTS OF VOLATILE ACID PRODUCED DURING GROWTH

	Standards			Distillate from glucose medium	Distillate from $\alpha$ -keto-glutarate medium
	Formic acid	Acetic acid	Propionic acid		
Fraction A	0.1250	0.2486	0.2860	0.2447	0.2183
Fraction B	0.1526	0.2322	0.2523	0.2380	0.1940
Fraction C	0.2300	0.2612	0.2374	0.2760	0.2360

Further evidence that acetate is an intermediate in glucose oxidation was obtained when it was found that cells harvested after four days' growth in an aerated liquid medium had almost completely lost their ability to oxidize acetic acid. It follows therefore that if acetate is an intermediate it should accumulate under these conditions of growth. This was found to be the case and about 30 times as great a concentration of acetic acid was found in the supernatant from cells that had lost their ability to oxidize acetic acid as in the supernatant from cells that still had the ability to oxidize this compound.

Since one-third of the oxygen consumed during the degradation of glucose would be used in converting acetic acid to carbon dioxide and water, if acetate is an intermediate, cells that cannot oxidize acetate should use only two-thirds as much oxygen as cells that can oxidize this compound. This was found to be true (Table III). The average value for normal cells is the value obtained from the data presented previously (7).

From Table III it can be seen that when glucose is oxidized by cells that have lost the ability to attack acetic acid, the amount of oxygen consumed is almost exactly the amount necessary to convert glucose to acetate, thus confirming the role of this compound as an intermediate in glucose oxidation.



TABLE III  
OXIDATIONS BY CELLS UNABLE TO ATTACK ACETATE

	Substrate	Oxygen uptake, $\mu$ l.
Average value, normal cells	Glucose	273
Observed value for cells unable to oxidize acetate	Glucose	179
Calculated value for cells unable to oxidize acetate	Glucose	182
Observed value for cells unable to oxidize acetate	Acetate	22

### Discussion

Yudkin (10) explained enzyme adaptation with the theory that "all examples of enzyme production are cases of increase in enzyme and none are instances of the formation of completely new enzyme" and further that "it is clear that the adaptive enzyme is produced from a precursor or precursors . . . it is assumed that an equilibrium exists between such precursors and the formed enzyme". According to Yudkin the combination of the enzyme with substrate would result in a disturbance of the equilibrium and more enzyme would be formed from the precursor in order to restore it. It therefore follows that within the physiological limits of the organism, an increased amount of substrate would result in the production of a greater amount of enzyme. This deduction is supported by the work of Kertesz (3) who found this to be true of invertase production by *P. glaucum*. Stanier's theory of simultaneous adaptation contradicts this work for it assumes that when a substrate is attacked by an adaptive enzyme the same amount of enzyme will be produced regardless of the amount of substrate present in the growth medium so that when cells are transferred from conditions where the compound in question is a metabolic intermediate, and therefore present in only very small amounts, to conditions where the same compound is present as parent substrate and in much larger quantity, no increase in amount of enzyme will occur.

In our studies it was found that, of the intermediates that theoretically should be identifiable by simultaneous adaptation, only those that were probably separated by only one enzyme reaction from the parent substrate reacted in accordance with Stanier's predictions. That is, cells grown on a glucose medium could attack gluconic acid without a period of adaptation, but there was a period of adaptation before they attacked 2-ketogluconic acid. Cells grown on pyruvic acid would attack acetic acid without a period of adaptation but cells grown on glucose had a period of adaptation before either pyruvic or acetic acids were oxidized.

Another limitation to the theory of simultaneous adaptation is that, if a pair of compounds are in enzymatic equilibrium, growth on a medium containing either of the compounds results in the production of cells that can immediately attack both compounds. The same examples as discussed above

will serve to illustrate this point. Cells harvested from a glucose medium are found to oxidize gluconic acid immediately at maximum rate and cells harvested from a gluconic acid medium were also found to attack glucose at maximum rate. According to the theory of simultaneous adaptation, glucose would thus be an intermediate in gluconic acid oxidation. Similar results were obtained for cells grown on either pyruvic or acetic acids when they were tested on either pyruvate or acetate and, according to the theory of simultaneous adaptation, one would be forced to conclude that acetic acid was oxidized by way of pyruvate.

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## AN IRREVERSIBLE GENE-INDUCED PLASTID MUTATION<sup>1</sup>

BY T. J. ARNASON<sup>2</sup> AND G. W. R. WALKER<sup>3</sup>

### Abstract

When plants of a variegated barley are self-pollinated, they produce few variegated and many albino offspring. In different years the proportion of albino plants has ranged from 80.2 to 93.1% of the total population. Seed from heads having much green tissue gave rise to a much larger proportion of variegated plants than did seed from heads with more white tissue. Maternal inheritance of plastids is probably the cause of this difference. In crosses  $F_1$  plants are green, variegated, or albino if the ♀ parent is variegated, but if the ♀ parent is green all the progeny are green. The albino plastids thus apparently do not mutate back to normal in the presence of the normal gene. In some  $F_2$  populations deviation from a ratio of 3 green : 1 others is insignificant, in other populations significant deviations, attributed to irregularities of plastid mutation and segregation, occur.  $F_2$  results support the hypothesis that a single pair of genes affecting plastids is segregating in hybrids. The normal (green) gene is dominant if "green" proplastids are present in the egg but not dominant if the proplastids are all "white". From cytological observations on sperms and eggs as well as from the genetic results, it is considered likely that direct plastid transmission to zygotes is exclusively from the female parent.

### Introduction

Since plastids can reproduce their kind they may be able to maintain, to a limited degree, independence of nuclear genes. Such independence is shown when there is maternal inheritance of mutated plastids. In a strain of variegated barley described earlier (1) and in the present paper, maternal inheritance of mutated plastids occurs. Inheritance is complicated, however, by continuing mutation of green to albino plastids in the presence of certain nuclear genes. Consequently selfed plants of the variegated strain produce no green offspring, even though eggs formed in green tissue patches must often contain "green" proplastids only. In crosses with green varieties, some segregation ratios are distorted by irregular plastid mutation and plastid segregation.

### Plastid Distribution in Variegated Plants

#### 1. Leaves

Freehand sections of variegated leaves were prepared by a modification of Froier's (2) method. The freshly cut sections, 20–50 $\mu$  thick, were examined immediately in a water mount. The addition of a small drop of iodine to each preparation increased the visibility of plastids.

In green mesophyll cells of variegated leaves, 37 to 46 green plastids, 5–7 $\mu$  in diameter, were counted (Fig. 1). In a transition zone between green and white tissues the number of green plastids was reduced to 1 to 21, and some of the plastids were also reduced in size. Cells with the number of normal plastids reduced contained 28 to 36 small bodies most of which had a diameter

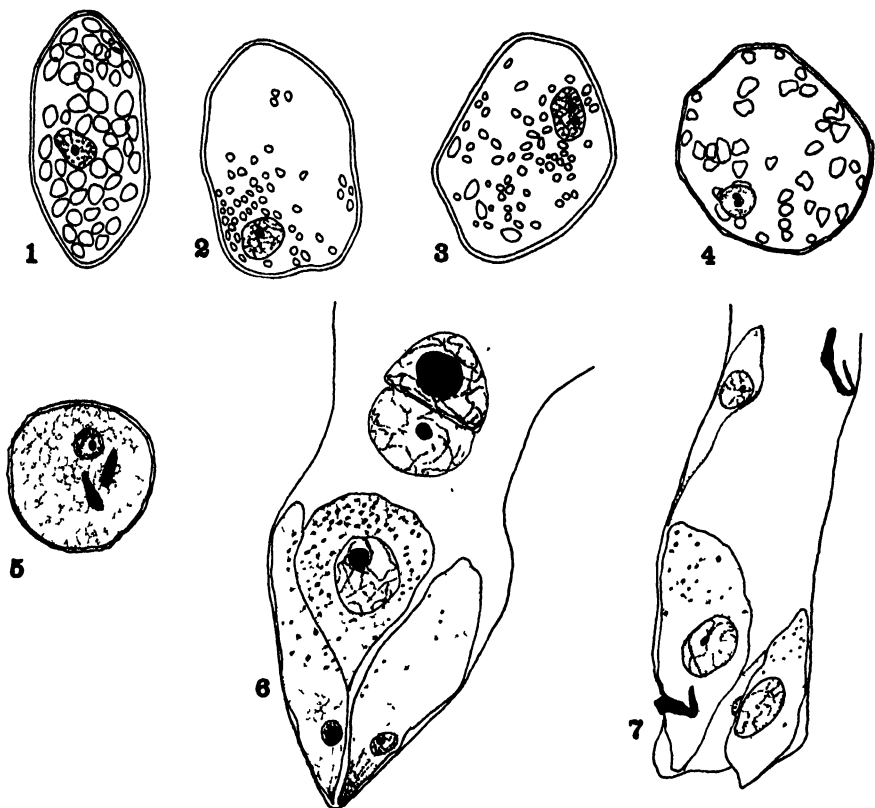
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of 1–3.5 $\mu$ . The staining reaction of these granules with iodine was similar to that of normal plastids. A cell having four green but slightly reduced plastids and a large number of the small granules is drawn in Fig. 3.



FIGS. 1 TO 7. All 540 $\times$ . Camera lucida drawings. FIG. 1. Normal green plastids, in outline, in mesophyll of barley leaf. FIG. 2. Small mutant plastids in white mesophyll cell of variegated leaf. FIG. 3. Mutant and green plastids in cell of variegated leaf. FIG. 4. Mutant plastids enlarged after detached leaf had been floated for 24 hr. in 10% sucrose solution. FIG. 5. Mature pollen grain containing two sperms. FIG. 6. Egg apparatus and fusing polar nuclei in barley embryo sac. Many proplastids in egg. FIG. 7. Sperm nucleus lying over egg, 24 hr. after pollination.

Except at some margins adjacent to green tissue no normal green plastids were observed in "white" cells but the number of small granules was increased, at least in some cells, to 40 to 50 per cell (Fig. 2). The small granules are doubtless mutated plastids, since, when white leaves are floated for 24 hr. on 10% cane sugar solution, they enlarge and deposit starch (Fig. 4).

If the cell lineage of adjacent cells in barley leaf cross sections were traced, one might find that many cell divisions had intervened between the common cell ancestor and the derived mature adjacent cells. For this reason one should not conclude that, if adjacent mesophyll cells differ markedly in plastid content, the cause must be something other than plastid segregation.

## 2. Gametes

Unfertilized eggs in embryo sacs contain, in the cytoplasm, many (more than one hundred) small round bodies that are probably proplastids. These are somewhat less abundant in synergids. No mature plastids are present.

It would be of interest to determine cytologically whether, at fertilization, a naked sperm nucleus enters the egg, or whether sperm cytoplasm enters also. Although sperm cells are known to be formed in male gametophytes of some Angiosperms (3), there is some doubt as to when the cell membrane bounding the individual sperms disappears. Decisive cytological evidence that sperm cytoplasm enters eggs appears to be lacking.

Sperms in pollen grains of barley have been studied in thin sections (7–10 $\mu$ ), stained in various ways. Nearly all the sperms appear to consist of naked, elongated nuclei. A slight "tail" is, however, visible at one end of some sperms, outside the deeply-staining nuclear mass (Fig. 5). Whether this lightly staining portion is cytoplasm or karyolymph is in doubt. No trace of sperm cytoplasm could be distinguished in sperms found near the egg after growth of pollen tubes (Fig. 7).

## Phenotypic Segregation in Progenies of Selfed Variegated Plants

In each year since 1942 over 500 offspring of selfed variegated plants have been classified according to phenotype as albino, variegated, or green. Over 80% (80.8 to 93.1%) of each population has consisted of albinos; the remaining plants, making up 6.9 to 19.2% of the total population have been, with few doubtful exceptions, variegated. Of a total of 7381 plants, 6488 (87.9%) were albino, 871 (11.8%) were variegated, and 22 (0.3%) were listed as green. All but one of the green plants were recorded in 1942, the classification being done on young plants with few leaves. In the last three years seedlings classified initially as green have been re-examined at intervals during the season and all of them have shown traces of variegation in later stages of growth. The green plants recorded in 1942 may have been, like these, faintly variegated.

The plants listed as variegated are by no means a uniform group; phenotypically there is a continuous range from white with a faint green stripe in one or two leaves to green with a narrow white stripe in some leaves. If, as was suggested earlier (1), nuclear genes are the same in the variegated and albino plants, then the phenotypic differences may most reasonably be attributed to plastid mutation and plastid segregation. The plastid or proplastid constitution of the fertilized egg will then be expected to have important effects on the phenotype of the leafy plant that develops from it. If the egg contains albino plastids only, the seedling is expected to be albino; If the plastids are mixed, or all green but susceptible to the mutagenic effects of the nuclear gene, the seedlings are expected to be variegated.

On the supposition that ovary and ovule tissues are probably white if the enclosing glumes are white, glumes of variegated heads were marked and the enclosed seeds tested. Results are recorded in Table I. The low proportion

TABLE I

NUMBERS OF ALBINO AND VARIEGATED SEEDLINGS FROM SEEDS FORMED IN WHITE AND VARIEGATED PORTIONS OF THE HEAD RESPECTIVELY

Glumes	Seed number	Seedlings	
		White	Variegated
White	99	98	1
Variegated	319	310	9

of variegated seedlings from the variegated portions of marked heads is doubtless a result of selecting, for glume marking, heads with higher than average amounts of white tissue.

To study differences between head progenies of variegated plants, heads were marked according to the percentage of white awns, the awns being used as an index of the amount of chlorophyll tissue in the heads. The results, recorded in Table II, are believed to be instructive. Heads having large

TABLE II

PROGENY OF INDIVIDUAL HEADS MARKED FOR PERCENTAGE OF WHITE AWNS

% White awns	No. of heads	Seedlings		
		Albino	Variegated	% Variegated
65 - 100	9	100	4	3.8
35 - 64	7	113	17	14.1
0 - 34	8	170	78	31.5

amounts of green tissue produce a much higher proportion of variegated offspring than do those that have less green and more white tissue. Direct plastid inheritance is the most probable cause of the observed differences. Eggs arising in green tissue inherit green plastids, some of which mutate prior to seedling emergence, thus giving rise to variegated plants. Eggs arising in albino tissue have "white" plastids only and give rise to albino plants. Although eggs arising in green tissue would be expected frequently to have all green plastids, no full green offspring are produced. Thus plastid mutation from green to white probably continues to occur prior to seedling emergence in every generation.

### Crosses Between Variegated and Green Regal Barley

As previously reported (1) Regal (green) ♀ × variegated ♂ results in all green  $F_1$  (one reported exception) while from the reciprocal cross green, variegated, and albino offspring are produced in no definite proportion.  $F_1$  results from several recent crosses including backcrosses are reported in Table III.

TABLE III

$F_1$  PHENOTYPES FROM CROSSES AND BACKCROSSES INVOLVING VARIEGATED (V) AND GREEN (G) PARENTS

Cross	$F_1$ AND BACKCROSS PHENOTYPES		
	Green	Variegated	Albino
G ♀ × V ♂	55	0	0
V ♀ × G ♂	56	43	151
V $F_1$ (V ♀ × G ♂) × G	13	0	2
V $F_1$ (V ♀ × G ♂) × V	8	0	1

The differences between  $F_1$  populations from the reciprocal crosses may most reasonably be explained on the assumption that direct plastid inheritance (maternal) may determine the phenotype. Since there is a large number of albino  $F_1$ , one may doubt that any albino plastids are converted to green by the genes contributed by the Regal parent. Albino plants could not be checked for hybridity since they all died in the seedling stage. That the variegated  $F_1$  are hybrids is attested by their green  $F_2$  offspring.

The  $F_2$  results from the Regal ♀ × variegated cross (Table IV) show a good fit to a 3 green : 1 of others ( $P = .3$  to  $.5$ ). In the reciprocal cross, however, the fit to a 3 : 1 ratio is poor ( $P$  is less than  $.05$ ), there being a considerable excess of variegated and albino segregates. Direct plastid inheritance is the most probable cause of the distorted ratio.

TABLE IV

$F_2$  SEGREGATION OF HYBRIDS FROM CROSSES BETWEEN REGAL (G) AND VARIEGATED (V) BARLEY

Cross	$F_1$ phenotypes	No. of $F_1$ plants	$F_2$ phenotypes			$\chi^2$ for 3 : 1 ratio	$P$
			Green	Variegated	Albino		
V ♀ × G ♂	Green	47	681	255	46	17.3	< .01
	Variegated	24	270	89	28	5.56	.01-.05
G ♀ × V ♂	Green	38	1322	347	113	0.60	.3-.5
Backcross $F_1$ V(V×G) ♀ × G ♂	Green	7	169	0	0	—	—
	Green	6	93	31	7	1.12	.20-.30
$F_1$ V(V×G) ♀ × V ♂	Green	8	133	45	7	0.96	.3-.5

In the backcross V  $F_1$  ♀ × G ♂ a 1 : 1 ratio of  $WW$  and  $Ww$  plants is expected if one gene pair is segregating. Of 13 green backcross plants obtained (Table III), seven bred true for green (Table IV) while six gave, when selfed, progenies approximating the expected 3 green : 1 others ( $\chi^2$  for 3 : 1 ratio based on bulked results of backcross  $F_2$  is 1.12).

When  $V F_1 \varnothing$  are backcrossed to  $V \sigma^7$  all the green offspring are expected to be heterozygous. The eight green backcross plants obtained (Table IV) all gave, when selfed, segregating progenies ( $\chi^2$  for 3 : 1 ratio calculated from bulked results is 0.96). The segregation of the backcross hybrids is in good agreement with the monofactorial interpretation.

Almost exactly one-third of the green  $F_2$  plants bred true (Table V), while two-thirds yielded segregating progenies. This result is in accord with the hypothesis that a single gene pair  $Ww$  is involved. The gene for green appears to be dominant when only "Green" plastids or proplastids are present in the egg.

TABLE V  
 $F_2$  POPULATIONS FROM GREEN (G) AND VARIEGATED (V)  $F_2$  PLANTS

Cross	$F_1$ phenotype	$F_2$ phenotype	No. of $F_1$ plants	$F_2$ phenotypes			$F_2$ geno- type	Deviation from expected $F_2$ ratio	$P$
				Green	Variegated	Albino			
$G \varnothing \times V \sigma^7$	G	G	133	3164	0	0	$WW$	0	1.0
	G	G	279	3911	1483	176	$Ww$	266.5	<.01
	G	V	49	0	333	568	$ww$	0	1.0
	G	V	3	38	10	12	$Ww$	7	.01-.05
$V \varnothing \times G \sigma^7$	G	G	19	456	0	0	$WW$	0	1.0
	G	G	39	657	224	29	$Ww$	25.5	.05-.30
	G	V	11	0	19	221	$ww$	0	1.0
	G	V	1	22	0	2	$Ww$	4.0	.05-.30

That plastid mutations may occur in heterozygous individuals is indicated by the rather large deviations from expected 3 : 1 ratios in segregating  $F_2$  populations. Actual proportions approach a ratio of 2 green : 1 others in some groups. Four of 64 variegated  $F_2$  plants (6.2%) from green  $F_1$  produced green  $F_3$  progeny and therefore probably had the  $W$  (green) gene.

Although a good fit to a 12 : 3 : 1 ratio is apparent in some  $F_2$  populations, no similar tendency is observed in the  $F_3$ . When all the available data from crosses are considered the most reasonable explanation of the results is that in  $F_1$  hybrids there is one segregating chromosomal factor pair  $Ww$ ; that  $W$  cannot cause mutations of albino plastids to green but that  $w$ , even in heterozygous individuals, may cause mutation of some green plastids to white. Distorted ratios are attributed mainly to the irregularities of plastid segregation and transmission.

### Albino $\times$ Regal (Green) Crosses

Attempts to rear albinos to maturity following Spoehr's (6) method were unsuccessful. Crosses involving white tillers of variegated plants ( $\varnothing$ ) and Regal ( $\sigma^7$ ) have yielded only four viable seeds, all of which produced albino



seedlings. There is, thus, from these slight results, no evidence of direct plastid transmission by the male.

### Discussion

Inheritance of variegation in barley parallels in several respects that of the iojap character in corn (5). In both, plastid mutation occurs in the presence of the homozygous mutant gene. The plastid mutation rate is sufficiently low to permit at least some homozygous but variegated plants to reach maturity and to produce seeds. Mutated plastids do not revert to normal in the presence of the plus gene.

Since the barley plastid mutation is gene-induced, the plastid is to that extent like other cytoplasmic components and unlike most nuclear genes. Few genes are known to induce mutation of other specific genes. An exception is the *Dt* gene in corn, which affects the *a* locus (4).

The mechanism of induction of the plastid mutation is not known, but probably involves the production, by the mutant nuclear gene *w*, of a diffusible substance that induces a specific directed plastid mutation. An alternative suggestion that the mutant gene *w* fails to provide some substance essential for normal chloroplastid development is probably not correct since white plastids do not become green even though the normal green (*W*) gene is present; e.g., in *F*<sub>1</sub> plants of the *V* × *G* cross.

If the albino plastids were immediately responsive to controlling nuclear genes an investigation designed to determine the point at which chlorophyll synthesis is blocked might result in useful information. However, since the albino plastids can presumably be converted to normal only by reverse plastid mutation it would appear useless to search for a chemical substance that, when introduced into white cells, would enable mutant plastids to develop into functional green plastids.

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## A CONVENIENT METHOD OF OBTAINING ASCOSPORES FROM BAKERS' YEAST<sup>1</sup>

BY A. M. ADAMS<sup>2</sup>

### Abstract

The superiority of methods involving the use of sporulation media containing acetate, first introduced by Stantial and Elder, over several commonly employed methods is established. A new method for obtaining ascospores from bakers' yeast cultures is recommended involving the direct transfer of vegetative cells from a solid nutrient medium to a solid medium containing acetate. High yields of ascospores are consistently produced after seven days' incubation. This method should lend itself particularly to use in the preparation of ascospores for instructional work, and for genetic research in yeast, and may also find application in yeast taxonomy. The technique recommended is as follows: vegetative yeast cells are multiplied on tomato juice agar or on dextrose nutrient agar, and are then transferred to a solid sporulation medium containing 0.04% dextrose, 0.14% anhydrous sodium acetate, and 2% agar.

### Introduction

The method most commonly employed to induce ascospore formation in yeast (the term "sporulation" is commonly employed in the literature when referring to this phenomenon) is the "plaster block" or, as it is also called by some workers, the "gypsum block" method. Plaster of Paris is mixed with water and allowed to harden. Vegetative yeast cells are then spread thinly over the surface, which should be kept moist. With some modifications the procedure remains essentially the same as when first introduced by Engel (5) in 1872. Rees (14) in 1869 conducted studies in which he obtained ascospores from vegetative cells after their transfer to a substrate of vegetable tissue. More recently Gorodkova (6) employed successfully an agar medium containing only a small amount of glucose, beef extract, and sodium chloride. Similarly, McKelvey (12) was able to induce ascospore formation with a weak carrot extract agar. Mrak, Phaff, and Douglas (13) obtained ascospores from agar slant cultures of vegetative cells on a vegetable extract medium. Investigating this problem, Lindegren and Lindegren (11) employed several fruit and vegetable extract agars.

One of the first workers to study sporulation yeast, de Seynes (16) found ascospores were formed when a wine yeast was transferred from grape must of ordinary sugar content to a solution containing only a small amount of sugar. More recently, Wickerham *et al.* (21) have recommended the use of commercial mixed vegetable juices.

Stantial, and later Elder, working in the Department of Chemistry of the University of Toronto have done much interesting and valuable work, the greater portion of which has not been published. Stantial found that a dilute

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medium containing sugar induced sporulation (17), and later (18) that a dilute medium containing both a sugar and an acetate consistently induced sporulation in vegetative cells. She was able to establish an optimum relationship between the concentration of cells and the concentration of acetate in the sporulation medium. She found that as the pH of the sporulation medium was decreased the yield of asci was also reduced. Best yields of ascospores were obtained with pH values of about 8 in sporulation media.

In unpublished manuscripts Elder (2, 3, 4) confirmed and enlarged upon Stantial's findings. As a presporulation medium Elder recommends a broth made by filtering, neutralizing, and autoclaving a commercial brand of tomato juice. Cells cultured in flasks for 24 to 48 hr. in this broth are freed from the liquid by filtration or centrifugation and washed twice with sterile distilled water. The cells are then resuspended in sterile distilled water. After adjustment of the cell concentration with the aid of a haemocytometer, a volume of suspended cells is mixed with an equal volume of liquid sporulation medium, making a final concentration of 20 million cells to 2 mgm. dextrose and 15 to 20 mgm. sodium acetate.

The writer recently had occasion to investigate some of the above methods since he required a reliable source of ascospores for certain research on yeast, and in this connection the method of Stantial and Elder was given special attention.

### Materials and Methods

Yeast cultures referred to in the experiments were of the commercial bakers' yeast type. Stock cultures were obtained by means of dilution and plating of cells from packaged yeast. Unless stated to the contrary, the cells transferred to sporulation media were from 24-hr. subcultures.

The media most commonly employed in the experiments to follow were:

(a) Dextrose nutrient broth—prepared by adding 5% dextrose to Difco nutrient broth.

(b) Dextrose nutrient agar—prepared by adding 2% agar to the broth in (a).

Tartaric acid (0.5%) was added to (a) and (b) to inhibit any bacterial contaminants.

(c) Grape juice broth—juice of freshly crushed red grapes was filtered, clarified, and diluted to approximately 5% sugar content.

(d) Grape juice agar—prepared by adding 2 to 2.5% agar to (c).

(e) Tomato juice broth—commercial canned tomato juice (salted) was filtered and clarified.

(f) Tomato juice agar—prepared by adding 2% agar to (e).

(g) Liquid sporulation medium—prepared by dissolving 0.04% dextrose and 0.14% anhydrous sodium acetate in distilled water. This medium is essentially the same as that employed by Elder.

(h) Solid sporulation medium—prepared by adding 2% agar to (g).

The percentage of ascospores in a given sporulation culture was estimated by counting a total of 400 asci and vegetative cells. Only asci containing two, three, or four ascospores were included in such counts to avoid the possibility of mistaking a vegetative cell for an ascus containing but one ascospore.

During the repeated employment of the Stantial-Elder method for producing ascospores, certain modifications that increased the value of the method were developed. These were:

(a) Vegetative cells may be multiplied on a solid nutrient medium instead of in a liquid medium. This obviates the necessity of filtration to free the cells from the culture medium.

(b) Similarly, the liquid sporulation medium may be replaced by a solid medium (see (h) above). When the latter is employed, cells may be transferred directly to sporulation medium from solid nutrient medium without involving the numerical estimation and adjustment of cell suspensions.

(c) When liquid sporulation medium is employed, the concentration of cell suspensions may be adjusted by comparison with permanent density standards prepared and employed as in the estimation of bacterial cell numbers by the McFarland nephelometer technique. This method of estimating cell concentrations was found to be much more rapid than the haemocytometer method employed by Stantial and Elder.

## Experimental

### (a) Effect of Pretreatment

In this experiment the yeast was grown in nine different media from which the cells were transferred to solid sporulation medium. This transfer was direct except in the case of the liquid medium where filtration was necessary to obtain the cells. The results listed in Table I confirm the findings of

TABLE I  
ASCOSPORE FORMATION BY CELLS GROWN ON NINE MEDIA

Pretreatment media	Percentage of ascus formation on solid sporulation medium after:	
	6 days	10 days
Dextrose nutrient agar	80	85
Grape juice agar	55	60
Grape juice	55	65
Tomato juice agar (commercial)	80	85
Tomato juice agar*	66	75
Glucose tryptone extract agar*	60	65
Beef infusion agar*	58	60
Beef lactose agar*	50	50
Nutrient agar*	20	20

\* *Disco products prepared according to directions on containers.*

Stantial and Elder that cells grown on tomato juice give high yields of ascospores. Whereas low yields were obtained from those grown on nutrient agar, the other media gave yields comparable to that of tomato juice.

*(b) Effect of Certain Chemicals on Ascospore Formation*

The ability of nine chemicals to induce ascospore formation in sugar-free liquid medium was assessed. Five concentrations of each chemical were employed and each sporulation culture had a volume of 20 ml. containing one million yeast cells per ml. Erlenmeyer flasks of 250 ml. capacity were employed as containers for the 20 ml. volumes of sporulation culture. (Throughout the remainder of the experimental work these containers were used for liquid sporulation medium, unless otherwise specified.) Vegetative cells for use here were grown on dextrose nutrient agar medium. At the conclusion of the experiment, viability of the cultures was determined by standard plating procedure to determine whether the chemicals had been toxic to the cells.

Table II indicates that the acetate radical was responsible for inducing ascospore production. Higher yields of ascospores were obtained from cultures in which the concentration of acetate was that recommended by

TABLE II

THE EFFECT OF VARIOUS CONCENTRATIONS OF CHEMICALS UPON ASCOSPORE FORMATION

Compounds employed*	Percentage yield of asci					Viability
	0 185**	0 375**	0 75**	1.5**	3 0**	
Potassium acetate	70	68	65	68	68	+
Calcium acetate	72	58	48	41	41	+
Acetic acid	65	25	0	0	0	+
Barium acetate	0	0	0	0	0	Much reduced
Sodium acetate	78	77	64	57	35	+
Magnesium acetate	65	65	32	8	0	+
Sodium oxalate	0	0	Rare	0	0	+
Sodium citrate	0	0	Rare	0	0	+
Calcium sulphate	0	0	0	0	0	+

\* All chemicals were C.P. grade.

\*\* These figures represent the percentage composition of the anion in 100 ml. of the sporulation medium.

Elder (4). Yields obtained from sodium acetate were equal to or greater than those from any other compound tested. The failure to obtain ascospores with barium acetate may have been due to a toxic effect since the viability of the cells was found to be greatly reduced. Strontium acetate was tested in another experiment and proved highly effective at inducing ascospore formation with both liquid and solid medium. Stantial (18), in an effort to

find agents that would induce ascospore formation, used many different chemicals. She obtained the best yields from sodium and potassium acetates—the two acetates reported in her studies.

(c) *Effect of Cell Numbers in Liquid Sporulation Medium on the Yield of Ascospores*

Sporulation cultures were prepared in which the concentrations of acetate and dextrose were constant and in the proportions described in the section on Materials and Methods. The number of cells in each culture was varied so that the effect of various cell populations on sporulation could be assessed. Two cultures of commercial yeast were used. The percentages of asci present after 14 days' incubation are listed in Table III. The percentage yield of

TABLE III  
THE EFFECT OF CELL NUMBERS IN LIQUID SPORULATION  
MEDIUM ON YIELD OF ASCOSPORES

Millions of cells per 20 ml. of liquid sporulation medium	Percentage yield of asci after 14 days	
	Culture No. 1	Culture No. 2
5	2	66
10	37	60
20	40	70
40	44	30
80	25	10
160	2	3

asci in culture No. 1 was reduced above and below the concentration of 20 million cells in 20 ml. of sporulation medium. This was repeatedly shown to occur by Elder (4). But in culture No. 2 the percentage of asci did not drop appreciably in the lower concentrations, indicating that yeast strains vary in this respect.

(d) *Effect of Depth of Medium on Ascus Formation*

Ascospore formation was observed to take place readily in culture suspensions in 250 ml. flasks when the volume of the suspensions was 20 ml. or less, but when similar suspensions were incubated in 10 ml. amounts in test tubes very few ascospores developed.

Investigating further, varying amounts of a sporulation medium cell suspension were added to a series of test tubes (16 mm.  $\times$  150 mm.). As shown in Table IV, from tubes in which the depth of the medium was 9 mm. or less, a high yield of ascospores was obtained, whereas at greater depth the percentage yield rapidly decreased. The factor responsible for this effect has as yet not been determined.

TABLE IV  
THE EFFECT OF DEPTH OF MEDIUM IN CULTURE TUBES  
ON ASCUS FORMATION

Depth of liquid sporulation medium, cm.	Volume of liquid sporulation medium,* ml.	Percentage of asci formed
0.55	0.25	84
0.70	0.50	76
0.90	0.50	70
1.00	1.00	5
1.05	1.00	2
1.70	2.00	2
2.00	2.00	2
2.60	3.00	0
3.20	4.00	Rare
4.60	6.00	Rare
6.10	8.00	0
6.90	10.00	0

\* The exposed surface area in each instance was 2.0 cm<sup>2</sup>.

(e) *Effect of Removing Cells from Sporulation Medium Containing Acetate to Distilled Water*

Stantial (18), and Elder (4), found that cells first suspended in the sporulation medium for four to six hours, then washed and resuspended in water, yielded asci. This matter was further investigated here in an experiment designed to determine how short an exposure to acetate-dextrose medium is required to induce ascospore formation. Cells were suspended in the acetate-dextrose sporulation medium for varying intervals, washed and resuspended in distilled water, then incubated. The asci present were estimated after seven days' and 14 days' incubation. No appreciable difference in the yields observed on the two days was noted. Cells suspended in the medium for periods of one-half, one, two, three, four, and five hours gave yields of 9, 7, 11, 13, 12, and 14% asci, respectively. The three control flasks whose cells remained in the acetate-dextrose mixture throughout the incubation period gave an average yield of 60%.

(f) *Ascus Production by Cells from Cultures of Various Ages*

A comparison between the agar to liquid transfer method and the agar to agar transfer method was made with cultures from dextrose nutrient agar of a commercial baking yeast 1 day, 12 days, 22 days, and 36 days old.

Table V shows that the older cultures failed to produce as high a percentage of asci as did the young culture. Perhaps this may be explained by the fact that in the older cultures a greater number of dead or heavily granular cells would be present. The ascospores produced were in all instances normal in appearance, the asci being observed to contain two, three, or four ascospores. It is clear from this experiment that to obtain maximum yields of ascospores it is preferable to use fairly young cultures of vegetative cells. Elder (4) showed that cells from young cultures gave the most satisfactory yields.

TABLE V

THE PERCENTAGE OF ASCUS PRODUCTION BY CELLS OF VARIOUS AGES EMPLOYING SOLID AND LIQUID SPORULATION MEDIA

Age of vegetative cells	Solid sporulation medium			Liquid sporulation medium		
	Percentage of asci after:					
	6 days	10 days	14 days	6 days	10 days	14 days
1-day-old culture	62	65	75	56	60	60
12-day-old culture	22	20	25	10	15	15
22-day-old culture	20	20	20	15	13	15
36-day-old culture	5	5	5	Occ.	Occ.	Occ.

Incidentally, it will be noted in Table V that, in all instances, higher yields were obtained with solid than with liquid sporulation medium.

*(g) Comparison of Sporulation Media Containing Acetate*

In this experiment the complete Stantial-Elder method was compared with the following modifications: (1) cells were multiplied on solid nutrient medium and added to liquid sporulation medium, (2) cells were multiplied on liquid nutrient medium and added to solid sporulation medium, and (3) cells were multiplied on solid nutrient medium and added to solid sporulation medium. It is seen that (1) embraces modification (a) as described under Materials and Methods, (2) embraces modification (b), and (3) both (a) and (b).

The yields listed in Table VI show that these modifications when employed give yields quite comparable to those obtained with the Stantial-Elder

TABLE VI

THE EFFECT OF VARIED TECHNIQUE UPON THE FORMATION OF ASCOSPORES WITH SPORULATION MEDIA CONTAINING ACETATE

Medium on which cells were multiplied	Cells transferred to solid sporulation medium			Cells transferred to solid sporulation medium containing a trace of peptone			Cells transferred to liquid sporulation medium		
	Percentage of asci after:								
	6 days	10 days	14 days	6 days	10 days	14 days	6 days	10 days	14 days
Dextrose nutrient agar	70	72	80	85	80	90	50	64	60
Grape juice agar	75	75	80	85	83	90	66	70	72
Tomato juice agar (Difco)	70	74	80	80	85	85	63	66	70
Tomato juice agar (commercial)	73	70	75	80	88	90	72	68	73
Grape juice	62	65	65	70	70	75	50*	57*	60*

\* Complete Stantial-Elder technique employed.



technique. The addition of a trace of peptone to the solid sporulation medium gave the highest yields. The five types of media employed to multiply the vegetative cells had no great effect upon the yield of ascospores. This confirms the results in the experiment on pretreatment effects described under (a) in which these media were also employed.

*(h) Comparison of Methods Generally Employed for Inducing Ascospore Formation*

Cultures were prepared according to methods recommended by different authors and at present in general use for the development of ascospores. The yields of asci obtained in successive trials by these methods and the method developed in this work were averaged and are tabulated for comparison in Table VII.

TABLE VII  
THE RELATIVE FREQUENCY OF ASCI FORMED BY VEGETATIVE CELLS  
IN VARIOUS ENVIRONMENTS RECOMMENDED FOR  
INDUCING ASCOSPORE FORMATION

Sporulation medium or substratum	Percentage of asci present after 10 days at room temperature
Gorodkova	20
McKelvey	2
Gypsum block	<1
Gypsum block and peptone water	<1
Carrot slab	3
Liquid sporulation medium	75
Solid sporulation medium	85
Solid sporulation medium with added peptone	90

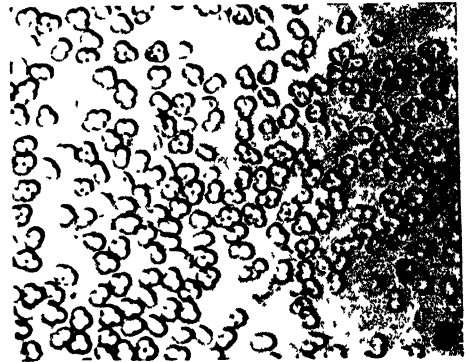
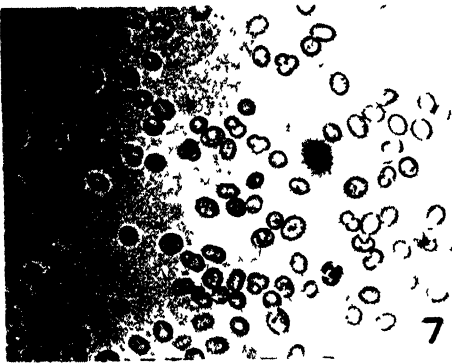
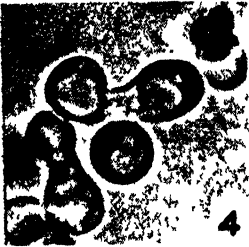
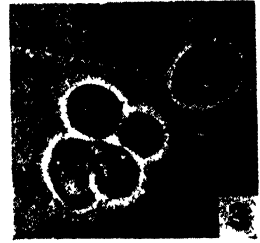
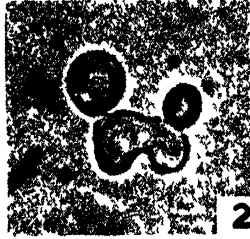
The medium developed by Gorodkova (6) contains only a small amount of glucose, beef extract, and sodium chloride in an agar base, thus providing a very limited supply of nutrients for the vegetative cells. This medium as is shown in Table VII ranked next to those containing acetate and was much more effective as a sporulation medium than the carrot slab, the gypsum block, or the weak carrot infusion medium of McKelvey (12). Figs. 7 and 8 show the development of ascospores upon solid sporulation medium containing acetate after four days' and 14 days' incubation. Many vegetative yeast cells can be seen in Fig. 7, whereas in the 14-day culture (Fig. 8) very few are encountered. Fig. 9 shows the appearance of asci and ascospores from solid sporulation medium under dark-field illumination.

FIGS. 1 to 6. Microphotograph of cells from mating medium in which ascospores were placed showing evidence of fusions occurring between ascospores and/or vegetative cells.  $\times 1200$ .

FIG. 7. Ascospore formation on solid sporulation medium after four days.  $\times 450$ .

FIG. 8. Ascospore formation on solid sporulation medium after 14 days.  $\times 450$ .

FIG. 9. Asci and ascospores after 14 days on solid sporulation medium. Dark-field illumination.  $\times 3000$ .





Although Petri dishes were found to be quite satisfactory as containers for the solid sporulation medium, test tubes afford a greater measure of protection from contamination during handling or storing. The preparation of sporulation cultures upon slants made with solid sporulation medium was found to give the same high yields of ascospores as did the Petri dish sporulation cultures. Test tube sporulation cultures have been previously prepared by Graham and Hastings (7) employing gypsum slants.

*(i) Observations on Fusions between Ascospores*

Winge (22) and Lindegren (10) have stated that fusions may occur between ascospores within an ascus, between haploid cells arising from different ascospores, and between ascospores and haploid cells, all three types of fusion resulting in the production of diploid cells.

Experiments were conducted to determine whether the ascospores formed in the presence of acetate would behave in a manner similar to that described by Winge and Lindegren. Employing a mating medium recommended by Lindegren (10) suspensions of ascospores (obtained from solid sporulation medium) were observed at intervals during a period of 24 hr. After 15 hr. a few fusions between ascospores were noted. During the next few hours these became somewhat more numerous, but the situation was confused by the vegetative budding of the cells in the suspension. Several typical fusions are illustrated in Figs. 1 to 6. It seems obvious that the ascospores formed by the method recommended here behaved in a like manner to that observed by Winge among ascospores produced on gypsum blocks.

## Discussion

Most of the methods recommended in the literature for inducing ascospore formation involve the use of plant tissue or tissue extracts. While it is possible that small quantities of acetate may have been present in these plant extracts, the first worker to recommend the addition of acetate was evidently Dr. Stantial. In 1939, Baltatu (1) working with the gypsum block method found that addition of acetic acid to the dilute grape juice medium that he poured over gypsum blocks increased the yield of ascospores.

But other factors than the presence of acetate may operate to stimulate the production of ascospores. Tanner (19) expressed agreement with Hansen (8) that a plentiful supply of air and a negligible amount of nutrient are required for good sporulation. Salle (15) states that sporulation can be brought about by culturing cells on starvation medium. Welten (20) spread washed yeast cells over glass plates and observed sporulation. It thus seems that adverse environmental conditions stimulate ascospore formation, which may account for the success of the plaster block method. On the other hand, Stantial was able to induce sporulation by adding orange, lemon, grapefruit, or tomato juice, and the juice expressed from lettuce to washed yeast cells in a water suspension. Lindegren and Lindegren (11) also had success with various combinations of vegetable and fruit juices. Evidently ascospores may be

formed in environments not unfavorable to growth. Thus from the literature it is evident that the environmental factors responsible for the formation of asci in yeast are not as yet fully understood.

A convenient and reliable method of obtaining ascospores is of some practical importance. Ascospores are essential for hybridization research leading to the development of improved yeast strains for industrial purposes. Also, since the characteristics of the ascus are of fundamental importance in the classification of yeasts, such a method would be of great value in identification of unknown strains. Henrici (9) recommends the plaster block method with McKelvey's medium (12) and Gorodkova's (6) as alternative methods but cautions that, "Spore formation with yeasts is, however, always uncertain, and a strain should not be considered non-sporogenous until several trials have been made with different methods". Lindegren (10), in a recent publication, studied the ability of 40 commercial bakers' yeasts to form ascospores. Here the highest yield reported was 50% asci; and of the remainder, 85% gave yields below 30%. Of these, 12% produced less than 1% of asci. The behavior of Lindegren's cultures in an acetate sporulation medium would be most interesting, as would also be the behavior of representatives of the *Torula* and *Zygosaccharomyces* yeasts. The possible effect that this improved method of obtaining ascospores may have upon yeast classification appears worthy of investigation.

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## STUDIES ON VARIATION IN PATHOGENICITY IN LEAF RUST OF WHEAT, *PUCCINIA TRITICINA* ERIKSS.<sup>1</sup>

BY A. M. BROWN AND T. JOHNSON<sup>2</sup>

### Abstract

In experiments designed to clarify the causes of the pathogenic changes that have recently been encountered in leaf rust of wheat, two factors were given consideration, (1) the possibility that nuclear exchanges might occur between the mycelia of different races in the uredial stage, and (2) the possible role of *Thalictrum* species in originating new rust strains. Infection studies with mixtures of races 9 and 15 and mixtures of races 5 and 9 did not produce any evidence that these races could interchange nuclei and thus initiate new pathogenic strains of leaf rust.

In greenhouse infection tests with native *Thalictrum* spp., aecia were produced on *T. dasycarpum*, pycnia only on *T. dioicum* and *T. polygamum*, while no infection of *T. venulosum* took place. In similar tests with the introduced species *T. glaucum* and *T. dipterocarpum*, abundant production of aecia occurred on both species. In out-of-doors inoculation tests with *T. dasycarpum* and *T. venulosum*, no infection of these two species took place, whereas, under the same conditions, heavy aecial production occurred on the introduced species *T. glaucum*.

Selfing studies in which *Thalictrum glaucum* was infected with known physiologic races have indicated that some races of leaf rust are homozygous, and others heterozygous for pathogenic characters. A culture of race 5 appeared to be homozygous, whereas a culture of race 3 was heterozygous, giving rise to races 3, 15, 32, 68, and three undescribed races. A culture of race 76 was heterozygous for both pathogenicity and urediospore color. Aeciospores of this race produced uredia of two different shades of yellow in addition to uredia of normal color. Aeciospores derived from teliospores collected in the field also gave rise to uredial strains of yellow spore color. Most of the yellow rust strains were decidedly low in pathogenic vigor.

### Introduction

Leaf rust of wheat (*Puccinia triticina* Erikss. = *P. rubigo-vera Tritici* (Erikss. & Henn.) Carl.) is one of the most highly specialized of the cereal rusts. In the third (1942) revision of the "International Register" of physiologic races of this rust, 129 races are described of which 68 have been encountered in North America. In recent years it has become evident that the physiologic race population in North America is by no means stable. It has been found (14) that certain wheat varieties that formerly showed considerable resistance to leaf rust in the field have become year by year more

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susceptible. This increase in susceptibility has been attributed to the widespread occurrence of race 128 and new biotypes of races 5 and 15. Other races that have been regarded (16) as important in this respect are 44, 58, and 126.

The question of the possible explanations of these pathogenic changes in leaf rust is one of obvious importance both practically and theoretically: practically, because an understanding of the process might suggest control measures; theoretically, because of the natural human interest in the mechanism of evolutionary processes.

On the basis of present knowledge, there appear to be three possible explanations for these pathogenic changes: (1) the function of alternate hosts, (2) the role of nuclear interchange in the uredial stage of the rust, (3) mutation. The work reported in the present paper was undertaken principally with the idea of exploring the possible function of the alternate host (*Thalictrum* spp.) and the role of nuclear interchange in the uredial stage as causes of variation in the leaf rust organism.

### The Possible Role of Nuclear Interchange in the Uredial Stage

It has been generally assumed that nuclear interchange in the uredial stage is not a factor in variation in the cereal rusts. This assumption is based on the knowledge that, in the uredial stage, the rust is in a dikaryotic condition in which the two associated nuclei divide conjugately and thereby supposedly remain in constant association with each other. The process of conjugate division is, therefore, thought to confer on the rust a stability somewhat comparable to that prevailing in plants with a diploid nucleus.

The experience of rust investigators appears to bear out this hypothesis. In *Puccinia graminis Tritici* Erikss. & Henn., physiologic races are frequently kept in pure culture for many years without any variant arising from them. Stakman *et al.* (21) refer to a uredial culture of race 1 of this rust that remained "constant pathogenically for more than 13 years". Evidence appears also to be lacking that physiologic races of this rust interchange nuclei in the uredial stage. Watson (22), in a study of competition between races 17, 19, 34, 56, and 147, does not report that he isolated from the mixed cultures any other races than those that entered into them. In nature, under epiphytotic conditions, there must occur between mycelia innumerable contacts that might furnish opportunities for nuclear exchange within a physiologic race or between physiologic races. Nevertheless, experience has shown that in the rust area of North America certain physiologic races of wheat stem rust, such as 56, 17, 38, and 32, recur year after year in much the same proportions. If nuclear exchange between the races of this rust were at all common, a much greater variety of races would be expected.

In *P. triticina*, it has been suggested by Ruth Allen (1), on the basis of cytological studies, that fusions may take place between two appressoria or two substomatal vesicles, which would obviously afford opportunities for

nuclear interchange between different mycelia. In a recent study, Rodenhiser and Hurd-Karrer (20) observed "fusion bodies" formed through anastomoses between germ tubes of the urediospores of wheat leaf rust germinating on nutrient agar, and noted a similar phenomenon on the surface of inoculated wheat leaves.

The above observations, though suggestive, do not prove that two or more mycelia of a rust can interchange nuclei. The only proof known to the writers that a dikaryotic rust mycelium can transmit a nucleus to another mycelium rests on the work of Brown (4, 5) who showed that dikaryotic mycelia of *Puccinia Helianthi* Schw., *Uromyces Fabae* (Pers.) de Bary, and *U. trifolii-hybridi* (W. H. Davis) Arth. were capable of dikaryotizing the haploid mycelia of their respective rusts.

If a dikaryotic mycelium can transmit a nucleus to a haploid (monokaryotic) mycelium, it would seem possible that two dikaryotic mycelia might interchange nuclei.

In an attempt to determine to what extent nuclear interchange occurred when dikaryotic mycelia of known races of leaf rust came into contact, urediospore inoculum composed of a mixture of two readily distinguishable races was increased by infecting a susceptible wheat (Little Club). The urediospores of the next generation were employed for the inoculation of the standard differential leaf rust hosts. In this manner, race 9 was mixed with race 15 and, separately, with a biotype of race 5, designated as race 5a. The cultures of each race originated from a single spore.

To ensure that the mycelia of the paired races would have ample opportunity of coming into contact, all inoculations with the mixed inocula were carried out by the use of an abundant spore supply and, in general, infections were closely spaced on the leaves. When the infection results on the differential hosts were recorded, particular care was taken to look for types of infection not characteristic of either of the paired races, e.g., type 3 or type 4 infections on the varieties Carina and Brevit.

The results of these tests were entirely negative—visible evidences of racial interactions were lacking and only the races entering into a mixture were reisolated from it. Fifteen reisolates from the pairings of races 9 and 15 were identified as either race 9 or race 15; and six reisolates from the pairings of races 9 and 5a were identified as one or the other of these two races. Despite the undoubted occurrence of numerous opportunities for the interchange of nuclei between the paired races, there is no evidence that such interchanges did occur.

These negative results do not prove that physiologic races of *P. triticina* are incapable of interchanging nuclei in the uredial stage. As nothing appears to be known about the effect on pathogenicity of such nuclear exchange, if it does occur, it is just possible that the nuclei in interlacing mycelia were reassociated without any visible pathogenic effect. It seems, however, reasonably safe to conclude that, as far as the races studied are concerned, nuclear interchange in the uredial stage is not of frequent occurrence.

## Races Originating from *Thalictrum* spp. Infected with Field Collections of Teliospores

As the sexual stage in the life cycle of leaf rust occurs on *Thalictrum* spp., it was thought that a study of the rust derived from that host might give some indication of the range of variability in leaf rust. Accordingly, two collections of teliospores gathered in the fall of 1946 at Winnipeg, Man., and Kapuskasing, Ont., were induced to germinate by subjecting them to periods of alternate wetting and drying during November. This material had not been subjected to freezing except possibly light frosts prior to the collection of the spores late in September. The teliospores collected at Winnipeg on Sept. 28 began to germinate about the middle of December and continued to do so until late in the following April.\*

Sporidia from the Winnipeg teliospores were sown on young plants of *Thalictrum glaucum* Desf.,\*\* which had proved to be a congenial host. Aeciospores derived from this host gave rise to 22 uredial isolates among which there were present the following races (the number of isolates of each race in brackets): race 1 (4); race 5 (1); race 15 (4); race 29 (2); race 76 (1); race 89 (3); race 113 (1); race 113a† (2); race 128a (4). The infection types produced by these races are shown in Table I.

Sporidia from the Kapuskasing teliospores were sown on *Thalictrum dipterocarpum* Franch. on which numerous aecia were produced. These gave rise to 14 isolates comprising the following races: race 1 (1); race 5 (1); race 15 (6); race 30 (1); race 37 (1); race 39 (1); race 126 (1); and two new races designated as C-1†† (one isolate) and C-2 (one isolate).

In these isolates of aecial origin there were present six races not found in the field in 1946 (the summer in which the above-mentioned teliospores were formed), namely races 30, 37, 39, 89, C-1, and C-2. On the other hand, certain races common in field collections were not represented in the isolates from aecia, such as races 3, 5a, 9, 15a, and 58, or were scantily represented, such as race 76.

In the rust of aecial origin, variation of a kind never encountered in uredial collections was observed, such as strains of red urediospore color, to which the wheat variety Little Club was resistant, and strains of yellow urediospore color. The latter occurred only in the rust derived from the teliospores collected at Kapuskasing, Ont. Four yellowish cultures, two of them bright yellow and two orange-yellow, were isolated. All were so pathogenically

\* The ability of leaf rust teliospores to germinate without undergoing a rest period at low temperature may not be unusual, for teliospores of races 1a, 5a, and 35, collected from greenhouse cultures on June 24, 1948, were induced to germinate a week later. They caused the infection of young *Thalictrum* plants and aecia were obtained in July.

\*\* The writers are indebted to Prof. C. W. Lowe, Department of Botany, University of Manitoba, for assistance with the identification of this host.

† Races designated by the letter "a" are virulent towards Hope, H-44, and the various wheats derived from them.

†† Isolates that did not closely approximate any of the 129 physiologic races described in the "International Register" were designated as C-1, C-2, etc., the letter C representing Canada. The infection types of these isolates are recorded in Tables I and II.

TABLE I

INFECTION TYPES PRODUCED ON DIFFERENTIAL HOSTS BY PHYSIOLOGIC RACES OF LEAF RUST, *Puccinia tritici*, ISOLATED FROM AECIAL CULTURES THAT ORIGINATED FROM TELIOSPORES COLLECTED AT WINNIPEG, MAN., AND KAPUSKASING, ONT. (ANY CULTURE THAT DEVIATED ONLY SLIGHTLY FROM SOME KNOWN RACE WAS ASCRIBED TO THE RACE IT RESEMBLED MOST CLOSELY)

Races isolated		Differential hosts								No. of times identified	
		Norka	Carina	Brevit	Webster	Loros	Mediterranean	Hussar	Democrat		Hope
From teliospores collected at Winnipeg											
	1	0	0-1	1	0-1	0-1	1	1+	1	x	4
	5	3	0-1	0-1	0-1	0-1	3	1	3	x	1
	15	0-1	0-1	1-2	1	1-2	3	2+	3	x	4
	29	3	2-3	2-3	x	3+	1	3	1	2-3	2
	76	0	x	3	x	3+	3+	2	3	x	1
	89	3+	2-3	2-3	2-3	3+	3+	3	3+	x	3
	113	3	2-3	2-3	2-3	2-3	3	2+	3	x	1
	113a*	3	2-3	2-3	2-3	2-3	3	2+	3	3	2
	128a	3	1-2	2-3	x-	2-3	1	3	1	3	4
From teliospores collected at Kapuskasing											
	1	0	0	0-1	0	0-1	0-1	1	1	x	1
	5	3	0-1	0-1	0-1	0	3+	1+	3+	x	1
	15	0	0	0-1	0-1	0-1	3	1+	3	x	6
	30	3+	2-3	2-3	3-	3	3+	1-2	3+	x	1
	37	3+	2+	2+	2+	3	1-2	2+	1	x	1
	39	3+	2-3	2-3	0-1	3	3	1-2	3+	x	1
	126	3	1	1	0-1	3	3+	1	3+	x	1
	C-1	0	1	1	3	3	2	1	2	x	1
	C-2	0	2	2	3	3	3	1	3	x	1

Definition of infection types in terms of host reaction:

0 = immunity; 1 = resistance; 2 = moderate resistance;

3 = moderate susceptibility; 3+ = susceptibility;

x = indeterminate reaction.

\* Cultures designated by the letter "a" are virulent to the variety Hope.

weak on Little Club and other wheats that they were lost before any congenial host could be found. Several isolates of normal color were also pathogenically weak on Little Club and were lost for that reason.

### Selfing Studies with Known Races

In *P. graminis Tritici* (12), and in *Melampsora Lini* (9), it has been shown that in most cases the inheritance of pathogenic characteristics proceeds in accordance with Mendelian laws. In crosses between races, certain infection types on a given host are dominant, others are recessive. In the selfing of a race of *P. graminis Tritici* (that is, its passage through the barberry and a study of the infection types in the next uredial generation), the race will

show segregation for dominant pathogenic traits in a heterozygous condition but not for recessive traits.

As nothing was known about the inheritance of the pathogenicity of leaf rust races, it was thought worthwhile to carry out selfing studies with some of the races in the hope of discovering the mode of inheritance of their pathogenic characteristics (infection types) on the differential hosts.

Several races of leaf rust, originating from monosporous cultures, produced teliospores in June, 1947. These teliospores were stored in a refrigerator at a temperature just above freezing until October, when they were taken out and alternately wetted and dried at room temperature. Early in November the teliospores began to germinate and were used to inoculate seedling plants of *T. glaucum*. Good pycnia of physiologic races 3 and 5a were obtained in December and these races were individually selfed by intermixing the nectar of their pycnia.

As the aecia that subsequently formed did not develop protruding cups, single-cup isolations were impractical but single-pustule isolations were readily obtained and the aeciospores were sown on seedling plants of Little Club. When the uredia developed, single-pustule isolates were made, increased, and cultured on sets of differential hosts.

#### *Selfing of Race 3*

Sixteen isolates of race 3 were studied, of which 11 were assigned to races already described in the International Register, and five were identified as one or the other of three new races. The reactions of the differential hosts to these races together with the times each race was isolated are shown in Table II.

A notable feature of the seven races isolated in this study was their exact resemblance to the parent race in their inability to rust the variety Norka and in the production of an x type of infection on the variety Hope. On Loros, Mediterranean, and Democrat, the type 3 infection of the parent race recurred in the progeny except for race 15, which produced a type 1 infection on Loros and race 68 which produced a type 1 on Mediterranean and Democrat. On Hussar there was also one deviation from the type 1 infection produced by the parent race, namely, the type 3 infection produced by race C-3. Infection types on Carina, Brevit, and Webster, however, showed considerable variation that is largely responsible for the variety of physiologic races derived from this selfing study.

From these results it may be concluded that the factors governing the infection capabilities of race 3 on Norka and Hope are in a homozygous condition, while those governing its infection capabilities on the other differential hosts are in a heterozygous condition.

#### *Selfing of Race 5a*

The results secured in the selfing of race 5a, as shown in Table II, indicate the possibility that the factors governing its infection capabilities on all the differential hosts are present in a homozygous condition. In this study, 29

TABLE II

INFECTION TYPES PRODUCED ON DIFFERENTIAL HOSTS BY PHYSIOLOGIC RACES OF WHEAT LEAF RUST, *P. tritici*, ISOLATED FROM THE SELFING OF RACES 3, 5a, AND 76. (ANY CULTURE THAT DEVIATED ONLY SLIGHTLY FROM SOME KNOWN RACE WAS ASCRIBED TO THE RACE IT RESEMBLED MOST CLOSELY)

Physiologic races isolated	Differential hosts									No. of times identified
	Norka	Carina	Brevit	Webster	Loros	Mediterranean	Hussar	Democrat	Hope	
From race 3 selfed										
3	0	1	2	1-2	3	3	1	3	x	4
15	0	0-1	1	0-1	1	3+	1-2	3	x	4
32	0	2	2+	2+	3	3	1	3	x	1
68	0	3	3	3	3	1	1	1	x	2
C-2	0	2+	2+	3	3+	3+	1	3+	x	1
C-3	0	2+	2+	2+	3	3	3	3	x	3
C-4	0	2-3	2-3	3	3	2-3	1	2-3	x	1
From race 5a selfed										
5a	3+	0	0-1	0-1	0-1	3+	1-2	3+	3	27
68	0	3	3+	3	3	1+	1+	1	x	1
105	3	0-1	3-	0-1	3	3	1	3	x	1
From race 76 selfed*										
1a	0	0	0	0	0	1	1	0	3	2
29a	3	2-3	3-	2-3-	3-	0-1	3	0-1	3-	2
101a	3	x	x	2	3	x	3	x	3	1
Light yellow	1	1	0-1	1+	0	0-1	0	1	1	5
Mars yellow	10a	3	3	3	3	0-1	0-1	0-1	3	3

\* For types of infection produced by race 76 see Table 1.

single pustule (uredial) isolates were identified as follows: 27 were assigned to race 5a; one to race 105, which is separated from the parent race by the susceptible reactions of Brevit and Loros and the indeterminate reaction of the variety Hope; and one to race 68, which is distinguished from the parent race by the reactions of all the hosts except Hussar. Norka, Mediterranean, and Democrat are all susceptible to race 5a but resistant to race 68. Hope is susceptible to the former race, but indeterminate in reaction to the latter. On the other hand, Carina, Brevit, Webster, and Loros are resistant to race 5a but susceptible to race 68.

This complete reversal in pathogenicity is difficult to explain and it may have been due to race admixture in the parent culture, in which case selfing might have been supplanted unwittingly by crossing, or by the selfing of an admixed race.

#### Selfing of Race 76

During March and April, 1948, pycnial cultures of race 76 were selfed and isolates originating from aecia were cultured and observed in a manner similar

to that just described. Altogether 13 uredial isolates were derived from the selfed cultures. Of these, eight developed uredia of yellowish color while the remaining five produced uredia of normal color. The cultures developing yellow uredia were subsequently segregated into two color groups, a light yellow and a deeper yellow—approximately the "Mars Yellow" of Ridgway (18). The light yellow cultures were all identified as race 1, while the Mars yellow cultures, all of them alike in pathogenicity, were identified as race 10.

From a study of the 13 isolates derived from the selfing of race 76, it may be seen (Table II) that the inability of race 76 to attack Norka was expressed in seven isolates, the uredia of two being normal in color while those of the other five were light yellow. The remaining six isolates attacked Norka heavily. The pathogenicity on Mediterranean and Democrat of all 13 isolates differed strikingly from that of the parent race. These varieties, susceptible to the parent race, were resistant to all 13 isolates. On the remaining differential hosts, Carina, Brevit, Webster, Loros, and Hussar, there was considerable variation in pathogenicity among the different isolates. Perhaps the most striking result encountered was the difference in pathogenicity of the two yellow rusts. The isolates that produced light-yellow uredia, and were identified as race 1, attacked weakly not only the differential hosts, but also Little Club, and consequently were maintained with difficulty. On the other hand, the isolates with deeper-colored uredia (Mars Yellow), although not as virulent as the isolates with normal colored uredia, nevertheless attacked heavily Norka, Carina, Brevit, Webster, Loros, and Hope.

Although the number of isolates observed was not large, the results obtained suggest that race 76 was heterozygous for pathogenicity and also for urediospore color.

### Reaction of *Thalictrum* Species and Their Possible Role in Nature

Despite the fact that several species of the genus *Thalictrum* have been proved to be more or less susceptible to *P. tritici* in greenhouse tests (8, 11, 17), the opinion held by most investigators is that *Thalictrum* species do not function actively in nature as alternate hosts of this rust (see Chester (7, p. 44); Johnson and Newton (13, p. 351)).

It is clear that, in North America, there is no such definite relationship between *Thalictrum* and wheat leaf rust as there is between *Berberis* and wheat stem rust. *Thalictrum* species produce no visible spread of leaf rust to adjacent fields of wheat. It is, nevertheless, possible that wheat leaf rust may occasionally produce aecia on one or another of the native species of *Thalictrum* and that the aeciospores may, in turn, cause infection on wheat. Such occasional infections would have little or no significance from the point of view of leaf rust epidemiology but might be an important factor in the origination of new leaf rust races. At all events, this aspect of the leaf rust problem should not be left out of consideration, particularly in view of the opinion expressed by Arthur (2, p. 181) that aecia of wheat leaf rust undoubtedly do occur on

*Thalictrum* species in North America although no collections of aecia have as yet been identified with that rust.

The determination of the true role of *Thalictrum* spp. in this connection is rendered more difficult by their susceptibility to several other rusts of the complex species *Puccinia rubigo-vera* (DC.) Wint. Cultural studies are therefore necessary before any given aecial infection can definitely be related to its graminaceous host.

In the Prairie Provinces of Canada, aecia do not occur abundantly on *Thalictrum* species, and the scanty evidence available indicates that such aecia as do occur belong to the *agropyrina* and *Agropyri* varieties of *P. rubigo-vera*. Fraser and Connors (10) attribute collections of aecia on *T. dasycarpum* Fisch. & Lall. and *T. venulosum* Trel. to *P. Clematidis* (DC.) Lagerh. = *P. Agropyri* Ell. & Ev. = *P. rubigo-vera Agropyri* (Erikss.) Arth. Bisby *et al.* (3) agree with this conclusion but with the reservation that the rust in Manitoba may possibly in some cases belong to var. *agropyrina*. One of the writers (T.J.) made two collections of aecia on *T. venulosum* at Winnipeg, Man., in 1935, that according to Arthur's Manual (2) were attributable to var. *agropyrina*. Neither collection would infect wheat. Aeciospores collected on this host at Clear Lake, Man., in 1947 likewise failed to infect wheat and were also attributed to variety *agropyrina*.

In 1948, attempts were made to determine the reaction to wheat leaf rust of several *Thalictrum* species both in the greenhouse and out-of-doors. In the greenhouse, germinating teliospores of several physiologic races were suspended over young plants of the native American species *T. dasycarpum*, *T. dioicum*,\* *T. venulosum*,\* and *T. polygamum* Muhl.\* and the two introduced species *T. dipterocarpum* and *T. glaucum*. Of these, the last two proved to be congenial hosts on which abundant aecia were formed. Of the native species, pycnia and aecia developed moderately well on *T. dasycarpum* and aeciospores taken from this host infected Little Club wheat. This species, however, appeared to be a less congenial host than the two introduced species. A weak development of pycnia was observed on *T. dioicum* and *T. polygamum*, but intermixing of the scanty nectar did not lead to aecial production. No infection occurred on *T. venulosum*.

Out-of-doors, in May, 1948, straws bearing viable teliospores of leaf rust of wheat were strewn in woodlots where the two native species *T. dasycarpum* and *T. venulosum* were plentiful, and in a sheltered garden where the introduced species *T. glaucum* had been planted. Weather was generally favorable for teliospore germination, and, on June 12, numerous aecia were observed on the plants of *T. glaucum*. The aeciospores from these were sown on Little Club wheat, which became infected. Aecia were again observed on the same plants

\* These species of *Thalictrum* were received through the co-operation of Dr. H. A. Senn, Ottawa.



on July 12 and again the aeciospores infected Little Club wheat. Throughout this period, the native species, *T. dasycarpum* and *T. venulosum*, remained free from infection.

These experiments indicate that the four native species tested (*T. dasycarpum*, *dioicum*, *polygamum*, and *venulosum*) are definitely less congenial hosts of wheat leaf rust than are the two introduced species *T. glaucum* and *T. dipterocarpum*. Though not clearly proved by the experiments, it seems probable that all the species are more resistant when grown out-of-doors than in the greenhouse. In view of the fact that *T. dasycarpum* became infected and produced aecia in the greenhouse, there is some possibility that this species might bear aecia out-of-doors. Its failure to become infected in the out-of-doors trial mentioned above does not prove that it cannot become infected.

It appears to the writers that the status of native *Thalictrum* species with regard to leaf rust of wheat is by no means settled. It seems particularly necessary to study further the reaction to leaf rust of *T. dasycarpum*, *T. dioicum*, and *T. polygamum*.

### Discussion

The most important problem confronting investigators of the cereal rusts is probably that of determining the causes and mechanism of variation, particularly variation in pathogenic potentialities. In the case of any rust that possesses an alternate host, it is generally assumed that variation occurs during the sojourn of the rust on that host. In assessing the situation with regard to leaf rust of wheat, one is confronted with the fact that it is not known with certainty whether or not an alternate host functions in the propagation of the rust. It is reasonably certain that an alternate host does not cause any widespread dissemination of this rust. It is not at all certain that an occasional aecial pustule on an alternate host may not occur; and, if it occurs, there exists a possibility that it may give rise to a new physiologic race of the rust.

Although species of the genus *Thalictrum* are generally regarded as the alternate hosts of leaf rust, it may well be that certain species of other ranunculaceous genera are equally functional. It has been shown (6) that, in Siberia, *Isopyrum fumarioides* bears aecia of wheat leaf rust. The related plant *I. biternatum* occurs in North America, but, as Mains (17) was not able to infect this species in greenhouse tests, there is no reason to suppose that it functions as an alternate host. Nevertheless, further studies should be carried out on the reaction to leaf rust of this and related plants, including species of genera known to be hosts of the varieties *Agropyri* or *agropyrina* of *P. rubigo-vera*.

If it could be proved definitely that the aecial hosts of *P. triticina* do not function as alternate hosts under natural conditions, there remains only the possibility that variation in this rust takes place in the uredial stage. It

seems not at all unlikely that a rust that does not utilize an alternate host might develop an alternative mechanism of variation. In this connection two possible mechanisms come to mind, i.e. (1) interchange of nuclei between different clones and (2) mutation.

The failure to secure evidence, in the present paper, for interchange of nuclei between races of leaf rust should not be regarded as definitive evidence against the operation of that process. As for mutation, no active investigation has been undertaken but, in view of certain reports (15, 19) of mutation in this rust, it is probable that mutation occurring in the uredial stage is one of the sources of variation. If any adequate understanding of the origin of variation in leaf rust is to be achieved, it is essential that further study be given to both the above mentioned processes.

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# INHERITANCE OF PATHOGENICITY AND UREDIOSPORE COLOR IN CROSSES BETWEEN PHYSIOLOGIC RACES OF OAT STEM RUST<sup>1</sup>

BY T. JOHNSON<sup>2</sup>

## Abstract

In the  $F_1$  generation of crosses between physiologic races 7 (orange) and 11 (red) of *Puccinia graminis* Pers. var. *Avenae* Erikss. and Henn., the medium sized uredia (type 2 infection) formed by race 11 on the oat variety White Tartar were dominant to the large (type 4) uredia of race 7, and the small (type 1) uredia of race 7 on the variety Richland were dominant to the type 4 uredia formed by race 11. On the varieties Sevnothree and Joannette Strain, the  $F_1$  hybrids produced the same type of infection as did the "maternal" parent race, that is, hybrids from race 7  $\times$  race 11 crosses produced a type 4 infection like race 7, and reciprocal hybrids produced a type 1 infection like race 11. These facts led to the suggestion that the cytoplasm of the maternal parent race influenced the infection type of the  $F_1$  hybrid on these two oat varieties.

A study of the  $F_2$  generation of the cross race 11  $\times$  race 7 showed that on the varieties White Tartar and Richland the dominant and recessive infection types appeared in a ratio of 9 : 7, which suggests that their inheritance is governed by two pairs of complementary genes. The distribution of physiologic races in  $F_2$  conforms to this assumption and indicates that the genes governing infection types on these two varieties associate at random to produce physiologic races 1, 11, 3, and 4. These races occurred in  $F_2$  in a ratio of 31 : 20 : 22 : 12, as compared to an expected ratio of 27 : 21 : 21 : 16. On the variety Sevnothree, 84 of 85  $F_2$  cultures produced type 1 uredia, in this way resembling the maternal parent, race 1, and the maternal grandparent, race 11. One  $F_2$  culture, only, produced the type 4 uredia characteristic of the paternal grandparent, race 7. It is concluded from this study, and from crosses between races 1 and 2, that the maternal (cytoplasmic) influence evidenced in the  $F_1$  generation persists in  $F_2$  and  $F_3$ .

In  $F_1$ , the red urediospore color of race 11 was dominant to the orange color of race 7. The distribution in these two color classes in  $F_2$  and  $F_3$  suggests that the inheritance of urediospore color is governed by a single pair of genes.

In crosses between physiologic races of *Puccinia graminis* Pers. var. *Tritici* Erikss. & Henn., studies have been made of the inheritance of pathogenicity and urediospore color in the  $F_1$ ,  $F_2$ , and  $F_3$  generations of certain crosses (2, 4, 5, 6, 7). No comparable studies have, as yet, been recorded for *P. graminis* Pers. var. *Avenae* Erikss. & Henn., although crossing results, confined to the  $F_1$  generation, have been reported (3). As some information is now available on the inheritance, in the  $F_2$  and  $F_3$  generations, of urediospore color and of certain pathogenic characteristics, it is the purpose of the present paper to present these results and, as far as possible, to interpret them.

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## A Cross Between Race 7 and Race 11

 $F_1$  GENERATION

The cross under consideration was one between race 7 and race 11—formerly described as race 10a (3). The infection types produced by the two races are illustrated diagrammatically in Fig. 1. In addition to differences in pathogenicity, the two races differed in the color of the urediospores. Race 11 produced uredia of the usual red color characteristic of oat stem rust, whereas race 7 produced uredia of a bright orange color. Before the cross was made, both parental races had been subjected to selfing studies from which it was known that race 11 was homozygous for color of uredia and for the infection types produced on the differential varieties White Tartar, Richland, and Joannette Strain, whereas race 7 was homozygous for color of uredia and for infection types on White Tartar and Joannette Strain but heterozygous for its infection type on Richland. The progeny of race 7 consisted predominantly of race 7, which produces small round uredia on Richland, but there were present also a number of cultures of race 6, which produces large linear uredia on this variety.

TABLE I

INFECTION TYPES OF RACES 7 AND 11 OF *P. graminis* VAR. *Avenae* AND OF THE  $F_1$  PROGENY OF CROSSES BETWEEN THESE TWO RACES

	White Tartar	Richland	Joannette Strain and Sevnothree
Parent race 7	(4)	(1)	(4)
Parent race 11	(2)	(4)	(1)
$F_1$ , 7 $\times$ 11 (7 crosses)	(2)	(1)	(4) = race 2 red
(9 crosses)	(2)	(4)	(4) = race 8 red
$F_1$ , 11 $\times$ 7 (6 crosses)	(2)	(1)	(1) = race 1 red
(4 crosses)	(2)	(4)	(1) = race 11 red

A total of 26 crosses were made between the two races (Table I). In the 16 crosses, race 7 orange  $\times$  race 11 red, pycniospores were transferred from haploid pustules of race 11 to haploid pustules of race 7, and the hybrid aeciospores therefore arose from haploid mycelia of race 7 that had received pycniospore nuclei of race 11. Of these 16 crosses, seven produced race 2 red and nine produced race 8 red. Of the 10 crosses in which pycniospores were transferred in the reverse direction, six produced race 1 red and four produced race 11 red. The occurrence of two races in each case was due to the heterozygosity of race 7 orange for infection type on Richland.

In these crosses, red urediospore color was dominant to orange; the medium sized uredia (type 2) produced by race 11 on White Tartar were dominant to the large uredia (type 4) produced by race 7; and the small uredia produced by race 7 on Richland (type 1) were dominant to the large ones produced by

race 11. In each cross, the infection type on the variety Joannette Strain resembled that of the maternal parent race, i.e., the race from whose haploid mycelia the aecia were formed. The inheritance of the infection types expressed on this variety is further discussed elsewhere in this paper.

### PROGENY STUDIES IN $F_2$ AND $F_3$

Only one of the 26  $F_1$  hybrids obtained in the above-mentioned crosses was submitted to further study. This hybrid, a culture of race 1 from the cross shown in Fig. 1, produced teliospores that germinated and caused infection on several barberry plants. The study of the distribution of rust character-

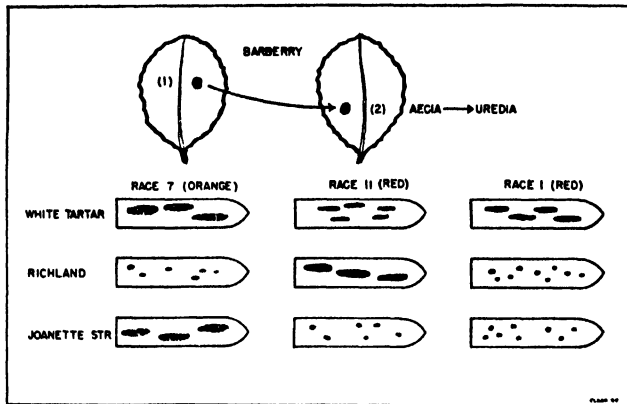


FIG. 1. Diagram illustrating the cross race 11 (red)  $\times$  race 7 (orange). Above—barberry leaves, as drawn when the cross was made, showing that nectar was transferred from one haploid pustule of race 7 to a haploid pustule of race 11. The hybrid aeciospores formed in pustule (2) gave rise to the  $F_1$  uredial generation, race 1 (red). Below—diagrammatic representation of infection types produced by the parent races and the hybrid race on the three differential oat varieties.

istics in the  $F_2$  generation was based on cultures established from 81 single aecia picked off at random from four barberry plants. From these aecia, 81 cultures of monoaecial origin were established. Of these, 77 produced a single physiologic race in each culture, whereas four produced two races in each. Thus the  $F_2$  generation studied comprised a total of 85 isolates.

The distribution of the physiologic races present in these isolates was as follows:

- Race 1—31 isolates (23 red; 8 orange)
- Race 3—22 isolates (13 red; 9 orange)
- Race 4—12 isolates (8 red; 4 orange)
- Race 8— 1 isolate (orange)
- Race 11—19 isolates (11 red; 8 orange)

Subsequently, three of the  $F_2$  isolates were selected for selfing studies designed to elucidate the inheritance of rust characters in the  $F_3$  generation, namely, one isolate of race 3 red, one of race 4 orange, and one of race 8 orange.

Each of these isolates was brought into the telial stage and the teliospores were, in due course, induced to germinate and infect barberry. All produced normal pycnia and aecia on the barberry. As in  $F_2$ , the  $F_3$  studies were based on aecia selected at random. The results of the  $F_2$  and the  $F_3$  studies are shown in diagrammatic form in Fig. 2.

### THE INHERITANCE OF UREDIOSPORE COLOR IN $F_2$ AND $F_3$

The data available on the distribution of urediospore color in the  $F_2$  and the  $F_3$  generation are summarized in Table II.

TABLE II

ACTUAL AND THEORETICAL DISTRIBUTIONS OF RED AND ORANGE UREDIAL CULTURES IN  $F_2$  AND  $F_3$  AND GOODNESS OF FIT TO A 3 : 1 RATIO

	Actual distribution	Theoretical distribution	
$F_2$ generation			
(1)*Red	55	63 75	$\chi^2 = 4.804$
Orange	30	21 25	$P = 0.05 - 0.02$
(2) Red	217	202 5	$\chi^2 = 4.153$
Orange	53	67 5	$P = 0.05 - 0.02$
$F_3$ generation			
(3) Red	53	51	$\chi^2 = 0.313$
Orange	15	17	$P = 0.7 - 0.5$

\*(1) = Data from 85 cultures derived from aecia selected at random (see Fig. 2).

(2) = Data from counts of red and orange pustules on oat leaves inoculated with mass transfers of aecia.

(3) = Data from selfing of an  $F_2$  culture of race 3 red (see Fig. 2).

Two sets of data were obtained, one from cultures derived from the random selection of aecia mentioned above, the other from a count of red and orange uredia on oat seedlings inoculated by mass collections of aeciospores. It may be noted here that the two sets of data bearing on color distribution in  $F_2$  are not in close agreement. The data derived from the 85 uredial cultures originating from a random collection of aecia show a ratio of 1.8 red to 1 orange, whereas data derived from a count of uredia on oat leaves inoculated with a mass collection of aecia show a ratio of 4.1 to 1.

The  $F_3$  data are limited to the selfing of one  $F_2$  culture, race 3 red, from which 68 aecia selected at random gave rise to as many uredial cultures. The ratio in this group of cultures was 3.5 red to 1 orange, a reasonably good fit to a 3 : 1 ratio.

It seems most probable from a consideration of the available data that the inheritance of urediospore color is governed by a single pair of genes that produce a 3 : 1 ratio in the selfing of any heterozygous culture. As both red and orange spores contain the yellow or orange cytoplasmic pigment, the

only difference between the two is that the red spores contain in the spore wall a pigment that is virtually absent from the orange spores. The pair of genes operative in the inheritance of color apparently governs the inheritance of this pigment.

### INHERITANCE IN $F_2$ AND $F_3$ OF INFECTION TYPES ON WHITE TARTAR AND RICHLAND

The essential facts concerning the inheritance of pathogenicity in the  $F_2$  and  $F_3$  uredial generations are shown in Fig. 2. Only those infection types present in the two parent races occurred in  $F_2$  and  $F_3$ ; no blending or intermediacy was observed.

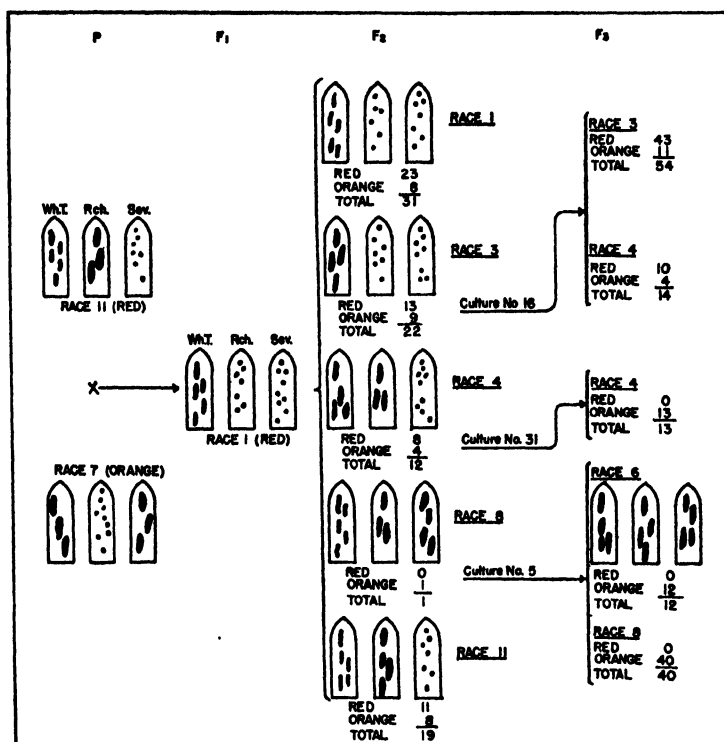


FIG. 2. Distribution of physiologic races in  $F_2$  and  $F_3$  of the cross race 11 red  $\times$  race 7 orange.

On White Tartar and Richland the large, type 4 uredia, suppressed in  $F_1$ , reappeared in  $F_2$ . The infection types on these two hosts were associated in all possible combinations: type 2 on White Tartar with type 1 on Richland to produce race 1 (as in  $F_1$ ). Type 4 on White Tartar with type 1 on Richland to produce race 3 (as in the race 7 parent). Type 2 on White Tartar with type 4 on Richland to produce races 8 and 11 (as in the race 11 parent). Type 4 on White Tartar with type 4 on Richland to produce race 4.



The last was the only combination not met with in the parents or the  $F_1$  generation.

On the host variety Joannette Strain and on Sevnothree, a selection from it, there was no comparable segregation for infection type, the infection type being that of the race 11 parent except for the appearance of the 4 type in one culture (race 8). The inheritance of the infection types appearing on these varieties is discussed elsewhere in this paper.

The distribution of infection types on White Tartar and Richland in  $F_2$  and  $F_3$  may be used to arrive at a factorial basis for the inheritance of pathogenicity on these varieties. Table III shows that for both White Tartar and

TABLE III

ACTUAL AND THEORETICAL DISTRIBUTIONS IN  $F_2$  OF TYPE 2 AND TYPE 4 INFECTIONS ON WHITE TARTAR, TYPE 1 AND TYPE 4 INFECTIONS ON RICHLAND, AND GOODNESS OF FIT OF THESE DISTRIBUTIONS TO A 9 : 7 RATIO

Infection type	Actual and theoretical distributions in $F_2$		
	Actual distribution	Theoretical distribution	
White Tartar			
(2)	51	47.8	$\chi^2 = 0.4895$
(4)	34	37.2	$P = 0.5 - 0.3$
Richland			
(1)	53	47.8	$\chi^2 = 1.2926$
(4)	32	37.2	$P = 0.3 - 0.2$

Richland the distribution of the contrasted infection types approximates a 9 : 7 ratio and therefore suggests that in each case the inheritance is governed by two pairs of genes that are complementary, the type 4 uredia appearing in the absence of the dominant genes of one pair or the other and in the absence of both pairs of dominant genes.

It may then be suggested that the genotypic constitutions of the parent races and the  $F_1$  hybrid are as follows:

	White Tartar infection type	Genotype	Richland infection type	Genotype
Parent—race 11	(2)	AA BB	(4)	cc dd
Parent—race 7	(4)	aa bb	(1)	CC Dd Cc or DD
$F_1$ —race 1	(2)	Aa Bb	(1)	Cc Dd

There are two reasons for supposing that the genotype of the race 7 parent for infection type on Richland was CC Dd or Cc DD rather than Cc Dd. One is that the selfing of the parent culture produced a distribution of 36 cultures with type 1 infection as against only four cultures with type 4 infection—a ratio much closer to the 3 : 1 ratio expected from either of the first mentioned genotypes than to the 9 : 7 ratio expected from the genotype Cc Dd. The other reason is that in the 26 crosses between races 7 and 11 there occurred 13  $F_1$  hybrids with type 1 infection on Richland and 13 with type 4 infection, which is in exact agreement with expectation if the genotype of race 7 were CC Dd or Cc DD. To account for the 9 : 7 ratio of these infection types on Richland in  $F_2$ , it would have to be assumed that the  $F_1$  culture, race 1, selected for selfing, possessed the genotype Cc Dd.

On the above assumptions the theoretical distribution of physiologic races in  $F_2$  (taking into consideration only the infection types on White Tartar and Richland) should be as shown in Table IV, i.e., 27, 21, 21, and 16 for races 1, 11, 3, and 4, respectively. Statistical analysis indicates that the actual distribution, i.e., 31, 20, 22, and 12, is a reasonably good fit and may be interpreted as giving support to the genotypic constitutions shown above.

TABLE IV

ACTUAL AND THEORETICAL DISTRIBUTIONS OF PHYSIOLOGIC RACES IN  $F_2$  BASED ON RUST BEHAVIOR ON THE VARIETIES WHITE TARTAR AND RICHLAND, THE THEORETICAL DISTRIBUTION BEING FOUNDED ON THE ASSUMPTION THAT TWO PAIRS OF GENES GOVERN THE EXPRESSION OF INFECTION TYPES ON EACH VARIETY

Infection types		Formulae*		Race	Actual distribution	Theoretical distribution	
White Tartar	Richland	White Tartar	Richland				
(2)	(1)	AB	CD	1	31	27	
(2)	(4)	AB AB AB	Cd cD cd } }	11 and 8	20	21	
(4)	(1)	Ab aB ab	CD CD CD } }	3	22	21	
(4)	(4)	Ab Ab Ab aB aB ab ab ab	Cd cD cd Cd cD cd Cd cD cd } }	4	12	16	$\chi^2 = 2.5450$ $P = 0.5-0.3$

\* A given formula in some cases includes several genotypes. For example, formula Abcd includes genotypes AAbbccdd and Aabbcdd.

The  $F_3$  data obtained from the selfing of  $F_2$  cultures of races 3 and 8 may be interpreted on the same assumption. Race 3 was homozygous for infection type on White Tartar but heterozygous for infection type on Richland, producing 54 cultures with type 1 (small) uredia on Richland and 14 cultures with type 4 (large) uredia. The actual ratio 54 : 14 is close enough to the theoretical ratio 51 : 17 to suggest a 3 : 1 ratio. On this supposition, the genotype governing the rust behavior of race 3 on Richland was either Cc DD or CC Dd. Race 8 was homozygous for infection type on Richland but heterozygous for that on White Tartar and produced an  $F_3$  generation made up of 40 cultures with type 2 (medium size) uredia and 12 cultures with type 4 (large uredia), a distribution very close to the 39 : 13 distribution expected if the ratio were 3 : 1. A genotype of either Aa BB or AA Bb would account for the distribution of the two infection types on White Tartar.

The only other  $F_2$  culture selfed, race 4, was, as would be expected, homozygous for the type 4 (large) uredia on both White Tartar and Richland.

It will be seen from Table IV that, whereas the original combinations of infection type 2 on White Tartar with type 4 on Richland that characterized the race 11 parent and type 4 on White Tartar with type 1 on Richland that characterized the race 7 parent recurred in  $F_2$  with about the expected frequency, the new combinations, type 2 on White Tartar with type 1 on Richland and type 4 on White Tartar with type 4 on Richland, were respectively too high and too low. To test the possibility that these discrepancies might indicate association between type 2 on White Tartar and type 1 on Richland, the actual frequencies obtained for the different combinations, i.e., 31, 20, 22, and 12, were subjected to the test of independence as described by Fisher (1). The high  $P$  value of 0.8 to 0.7 indicated no association and would therefore suggest that no linkage exists between any of the characters.

#### THE INHERITANCE OF INFECTION TYPES ON SEVNOTHREE\*

In considering the inheritance of the infection types of the parent races 7 orange and 11 red on the variety Sevnothree it is necessary to refer back to the results obtained in  $F_1$  from the crosses as previously described (3). Both parent races were proved by selfing studies to be homozygous for the infection types on Joannette Strain and the selection Sevnothree, which was derived from it. Race 7 orange produced exclusively large (type 4) uredia and race 11 (then referred to as race 10a) exclusively small (type 1) uredia.

It will be seen from the data presented in Table I that the progeny arising from the crosses and reciprocal crosses differed only in one respect, namely, in the infection type on Sevnothree. In all cases in which the hybrid aeciospores were formed in haploid mycelia of race 7 orange, the hybrid rust produced on Sevnothree large (type 4) uredia that were identical with the uredia

\* The variety Sevnothree, a selection from Joannette Strain, has replaced the latter as a differential host. Its reaction to the physiologic races discussed in this paper is identical with that of Joannette Strain. Any reference to infection types on one of these varieties applies equally to the other.

produced by the race 7 parent rust except for their color, which was red instead of orange. In the reciprocal crosses in which the hybrid aeciospores arose from race 11 haplonts dikaryotized by race 7 nuclei, the hybrid rust produced small (type 1) uredia similar to those produced by the race 11 parent rust.

In reporting these crosses (3) it was stated that "this phenomenon does not seem to be capable of explanation on a chromosomal basis, but can be readily explained if it is assumed that the pycniospore nucleus of the paternal race reaches the protoaecium of the maternal race unaccompanied by its cytoplasm, which, in any case, is very limited on account of the minute size of the pycniospores. Each hybrid aeciospore would then receive from the paternal race only a nucleus but from the maternal race a nucleus plus cytoplasm."

This phenomenon was first encountered in crosses between physiologic races of *P. graminis* var. *Tritici* and was there expressed in the infection types produced on Marquis wheat (5, 6, 7). Progeny studies with hybrid rusts displaying this supposed cytoplasmic effect revealed that it persisted in a large measure in the  $F_2$  generation (5, 6). Some variation, however, did exist in the infection types produced on Marquis by different  $F_2$  rust lines, which indicated a possible segregation of nuclear factors affecting rust behavior on Marquis, a segregation, however, balanced or obscured by the cytoplasmic effect.

One of the reasons for the present study was to determine the expression in the  $F_2$  generation of the supposed cytoplasmic effect observed in  $F_1$  on Joannette Strain and Sevnothree. In the study of the 85 cultures comprising the  $F_2$  generation, particular notice was taken of the infection type produced on the variety Sevnothree. As shown in Fig. 2, the  $F_2$  cultures, with one exception, were classified as one or another of races 1, 3, 4, or 11, all of which produced a type 1 infection on Sevnothree. In the one exception just referred to, an  $F_2$  culture with orange spores, large uredia were observed among the small uredia on Sevnothree. An isolation from the large uredia produced a pure culture of race 8 orange, which consistently produced large (type 4) uredia on Sevnothree, whereas an isolation from the small uredia gave rise to race 11 orange, pathogenically identical with the other culture except that it produced small uredia on this variety.

No explanation can be given as to the mechanism involved in this change from a type 1 to a type 4 infection; but that it was an irreversible change was shown by a selfing of the above-mentioned culture of race 8 in which the  $F_3$  cultures studied, 52 in all, produced the type 4 infection on Sevnothree.

In the other 84 cultures comprising the  $F_2$  population, there did not seem to be any visible difference in infection type on Sevnothree. The uredia conformed to type 1 infection except when the temperature of the greenhouse rose excessively during the early stages of pustule formation, in which case a considerable inequality in size was noted among the uredia produced on each leaf. This reaction to temperature, however, is not peculiar to the cultures under study but is characteristic of all races that produce the type 1 infection on Sevnothree.

In connection with the type 1 infection on this variety, there appears to exist between host and parasite a rather delicate balance that renders this infection type more subject to the influence of environment than the gene-controlled infection types on White Tartar and Richland.

The selfing of the two  $F_2$  cultures, race 3 red and race 4 orange (see Fig. 2), showed that the supposed cytoplasmic effect noted in the  $F_1$  and  $F_2$  generations persisted equally in the  $F_3$  generation. The infection types produced on Sevnothree in these studies were identical with those described above for the  $F_2$  generation.

### **Inheritance of Infection Type on Sevnothree in Crosses Between Races 1 and 2**

It was originally planned to study an  $F_2$  generation of the cross 7 orange  $\times$  11 red as well as the reciprocal cross 11 red  $\times$  7 orange. This study would have enabled a comparison to be made between an  $F_2$  generation derived from an  $F_1$  hybrid producing a 4 type infection on Sevnothree and an  $F_2$  generation derived from a reciprocal  $F_1$  hybrid producing a 1 type infection on that variety. Only the latter study, which has been reported above, was accomplished.

With a view to making a similar comparison, crosses were made between races 1 and 2, which resemble races 11 and 7, respectively, in the infection types produced on Sevnothree, i.e., race 1 produces a type 1 and race 2 a type 4 infection. The two races produced identical infections on the other two differential hosts, White Tartar and Richland, and were, as far as was known, pathogenically identical except for the difference in infection type produced on Sevnothree. They differed, however, appreciably in urediospore color. Race 1 produced uredia of normal red color, whereas race 2 produced grayish-brown uredia. Prior to crossing, nothing was known about the breeding behavior of race 1 but race 2 had been subjected to a selfing study that had shown it to be homozygous for color of uredia and for pathogenicity.

The results of the crosses and subsequent progeny studies are shown in Fig. 3. The  $F_1$  hybrid arising from the race 1 haploid pustule that received race 2 pycniospores (race 1  $\times$  race 2) was pathogenically identical with the race 1 parent but produced uredia of a slightly duller red color. The  $F_1$  hybrids arising from the three race 2 haploid pustules that received race 1 pycniospores (race 2  $\times$  race 1) were pathogenically the same as the race 2 parent but produced uredia of a rather light red color instead of the grayish-brown characteristic of the race 2 parent.

Progeny studies were carried out with two of the hybrids as is shown in Fig. 3. In neither case was any visible segregation for pathogenicity observed in the  $F_2$  generation. The selfing of race 1 gave rise to 23  $F_2$  cultures of which 12 arose from as many pustules of aecia and 11 from single aecia selected at random. These 23 cultures were pathogenically alike and conformed closely to race 1 but differed among themselves in the color of the uredia, 17 being classified as red and six as grayish-brown. The selfing of race 2—the hybrid

derived from pycnial pustule (1) of the cross race 2  $\times$  race 1—produced seven  $F_2$  cultures originating from seven different pustules of aecia. These appeared to be pathogenically alike and were all identified as race 2. Six of the cultures produced red uredia but one formed grayish-brown uredia similar to those of the grayish-brown grandparental race.

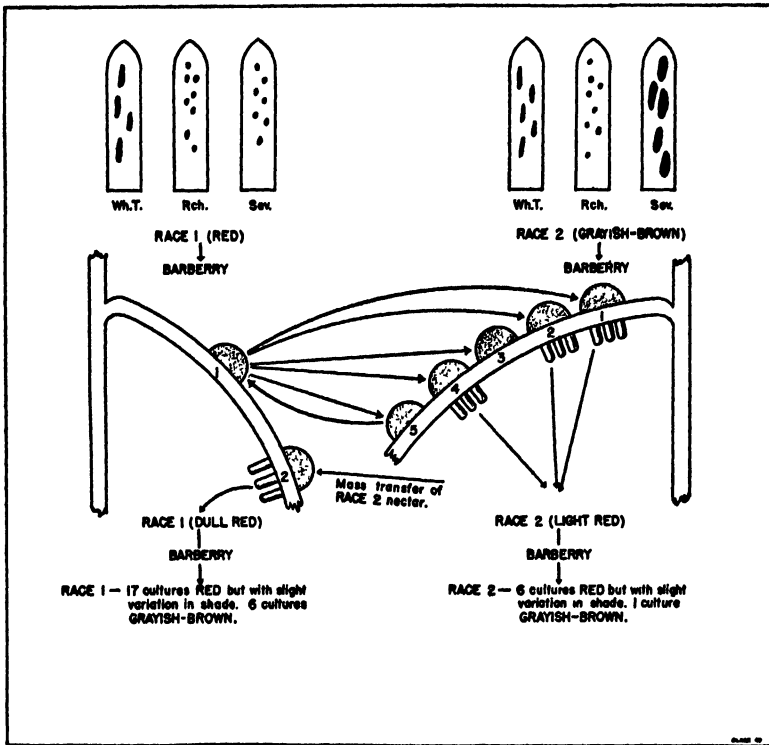


FIG. 3. Crosses between races 1 red and 2 grayish-brown and subsequent progeny studies. Nectar was transferred from pycnial pustule (1) of race 1 red to pycnial pustules (1), (2), (3), (4), and (5) of race 2 grayish-brown and a reciprocal transfer was made from pustule (5) of race 2 grayish-brown to pustule (1) of race 1 red. Pustule (2) of race 1 red received a mass transfer of nectar from several pustules of race 2.

### Discussion

It is established in the present paper that pathogenic characteristics in oat stem rust are inherited in much the same manner as in wheat stem rust (4). The ability of the rust to develop certain types of infection on given host varieties is conditioned by genes behaving in accordance with Mendelian laws. In the cross between races 7 and 11, the type 2 infection expressed on the variety White Tartar is a dominant character, the type 4 infection being the corresponding recessive character. Similarly, on the variety Richland, the type 1 infection is dominant to the type 4. In both cases, the inheritance in  $F_2$  was apparently governed by two pairs of factors complementary to each other.

As in wheat stem rust, there is evidence that not all pathogenic characters are under the governance of Mendelian, i.e., nuclear, factors. In wheat stem rust, it has been shown (5, 6, 7) that the inheritance of certain infection types expressed on the variety Marquis could be explained only on the assumption that the cytoplasm of the "maternal" parent race influenced the type of infection produced by the  $F_1$  hybrid. The selfing of the  $F_1$  hybrid showed, furthermore, that this influence persisted to a large degree in the  $F_2$  generation, which showed a decided, though not absolute, uniformity in the infection type on Marquis while at the same time there was clear evidence for segregation in the pathogenic characters expressed on certain other wheats. In the case of crosses between races of oat stem rust, it has been shown previously (3) that a similar influence of the maternal cytoplasm must be postulated to account for the inheritance in  $F_1$  of the infection types of races 7 and 11 on the variety Joannette Strain and its selection Sevnothree. In the present paper, evidence is presented to show that hybrids between races 1 and 2, which correspond respectively to races 11 and 7 in types of infection on Joannette Strain and Sevnothree, show in  $F_1$  the same behavior on these oat varieties as did hybrids between races 11 and 7.

It is now for the first time demonstrated that in oat stem rust the cytoplasmic influence affecting the infection types on these two varieties persists in the  $F_2$  generation in much the same manner as did the cytoplasmic influence expressed by wheat stem rust races on Marquis.

No new evidence is presented to explain the mechanism of this type of inheritance. It must be supposed either that there are factors of some sort that are transmissible through the cytoplasm of the maternal parent rust or that there are genic (nuclear) factors that operate differently in one cytoplasm than in another. In the latter case, the factors would not be cytoplasmic but rather nuclear factors influenced by the cytoplasm. Whatever the nature of these factors they are evidently not immutable. The occurrence, in one of the 85  $F_2$  cultures of the cross race 11  $\times$  race 7, of a clone with type 4 instead of type 1 infection on Sevnothree suggests a mutation in the factors governing the infection type on this host. That this mutation was no transitory change was shown by a selfing of this clone (culture No. 5 in Fig. 2) whose progeny displayed the new characteristic in all the 52  $F_2$  cultures studied.

The demonstration of the cytoplasmic inheritance of the type 1 and type 4 infections on Joannette Strain and Sevnothree in the crosses discussed in the present paper raises the question of whether the type 1 and type 4 infections on these varieties are in all instances similarly inherited. It is possible to arrive at some opinion in this connection by considering the various selfing studies carried out with physiologic races exhibiting these infection types. If, in a given physiologic race, the cytoplasm conditions pathogenicity and, if there is no appreciable effect through the agency of nuclear factors, it should follow that there would be no visible variation in infection type in the progeny. The chief difficulty in the way of this line of reasoning is that, in any strain of

rust for which cytoplasmic inheritance has not been demonstrated by reciprocal crosses, it is possible that uniformity in the progeny may be due to homozygosity of nuclear genes rather than to cytoplasmic control. But all races would not be expected to be homozygous for nuclear genes, and, if a number of races are studied and all are found to be homozygous for the characters in question, there would be some reason to suppose that cytoplasmic agencies rather than nuclear factors were at work.

To test this idea the available records of selfing studies with physiologic races of oat stem rust were examined with particular attention to the infection types produced on Joannette Strain. The results of this survey are summarized in Table V.

Of the 11 different clones of races 2, 6, 7, and 8, which produced type 4 infection on Joannette Strain, it will be seen that nine produced a uniform progeny in this respect, whereas two showed a tendency to produce type x infection in some or all of their progeny. Only one clone, race 6 orange No. 2, showed any real indication of segregation. In the case of the other clone that showed indications of type x infection in its progeny, race 8 No. 3, there was no apparent segregation but all isolates appeared to be on the border line between types 4 and x. It seems justifiable to conclude that, as far as the type 4 infection is concerned, there is no definite segregation of infection types.

With respect to the type 1 infection, the available information is more scanty and the evidence less clear. Only three clones were studied, one of race 3 and two of race 11. One of these, race 11 No. 1, showed no evidence of segregation. The second clone of race 11 gave rise to a progeny of 52 isolates identical in all respects except that 40 of them produced type 1 infection on Joannette Strain whereas 12 produced type x infection. This conforms closely to the 3 : 1 ratio, which might be expected from the governance of a single pair of Mendelian factors. The third clone studied, race 3, produced two isolates with type x infection out of a total of 25 isolates. In view of these results, it seems likely that inheritance of the type 1 infection is not wholly conditioned by non-nuclear agencies.

The inheritance of the third type of infection that occurs on Joannette Strain, type x, has not been studied to any great extent. Results of crosses of type x races with type 1 and with type 4 races have indicated a maternal inheritance of the type x infection (3). In Table V are recorded selfing studies with four clones of races that produced type x infection on Joannette Strain. Three of these, race 5 No. 2, race 10, and race 12, produced the type x infection in all their progeny—a fact that seems to show conclusively that the type x infection in these clones is not the result of heterozygosity for nuclear factors. The fourth clone, race 5 No. 1, produced type x infections in 95 out of 106 isolates and type 4 infections in the remaining 11 isolates. A possible interpretation is that nuclear as well as cytoplasmic factors affected pathogenicity in this clone.

As far as present available evidence permits a conclusion to be drawn concerning the inheritance of the infection types on Joannette Strain, it would seem



TABLE V

INHERITANCE OF INFECTION TYPE ON JOANETTE STRAIN IN THE SELFING OF OAT STEM RUST  
RACES NOT DERIVED FROM CROSSES

Race selfed	Infection on Joanette Strain	Races in progeny*	Infection types on differential hosts		
			White Tartar	Richland	Joanette Strain
2 No. 1	Type 4	2 (23)	2	1	4
2 No. 2	Type 4	2 (3)	2	1	4
2 orange	Type 4	2 (15)	2	1	4
2 gray	Type 4	2 (13)	2	1	4
6	Type 4	6 (14)	4	4	4
		7 (3)	4	1	4
		8 (12)	2	4	4
6 orange No. 1	Type 4	6 (38)	4	4	4
6 orange No. 2	Type 4	6 (33)	4	4	4
		13?(3)	4	4	4 to x
7 orange	Type 4	7 (43)	4	1	4
8 No. 1	Type 4	6 (7)	4	4	4
		7 (1)	4	1	4
		8 (15)	2	4	4
8 No. 2	Type 4	6 (2)	4	4	4
		8 (4)	2	4	4
8 No. 3	Type 4?	6?(6)	4	4	4 to x
		8?(8)	2	4	4 to x
3	Type 1	3 (15)	4	1	1
		4 (8)	4	4	1
		12 (1)	4	1	x
		13 (1)	4	4	x
11 No. 1	Type 1	11 (25)	2	4	1
11 No. 2	Type 1	10 (12)	2	4	x
		11 (40)	2	4	1
5 No. 1	Type x	2 (11)	2	1	4
		5 (91)	2	1	x
		10 (2)	2	4	x
		12 (2)	4	1	x
5 No. 2	Type x	5 (29)	2	1	x
10	Type x	10 (23)	2	4	x
12	Type x	12 (14)	4	1	x

*Number of isolates in parentheses.*

that the results of crosses and selfing studies with hybrids demonstrate that the type 4 and type 1 infection may be governed by cytoplasmic factors. The results of selfing studies indicate that in some clones, at least, nuclear factors may affect inheritance, particularly of the type 1 infection. The rather limited evidence available from crossing studies suggests that the inheritance of the type x infection may also be cytoplasmically conditioned but here, again, the selfing studies appear definitely to preclude the view that nuclear factors never enter into the inheritance of this infection type.

### Acknowledgments

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NATURAL PINE HYBRIDS IN ALBERTA<sup>1</sup>By E. H. Moss<sup>2</sup>

## Abstract

In central and northwestern Alberta, where the ranges of *Pinus contorta* var. *latifolia* and *P. Banksiana* overlap, there is abundant field evidence of hybridism between these species. Numerous trees have been found with cone and other characters more or less intermediate between those of the two species. These trees are generally found in proximity with, but rarely apart from, the putative parent species. Various diagnostic characters for the species are briefly evaluated in interpreting the polymorphic hybrid population. Characters of the mature seed cones are used to distinguish several pine types in the region and as a basis for a discussion of problems requiring further investigation.

## Introduction

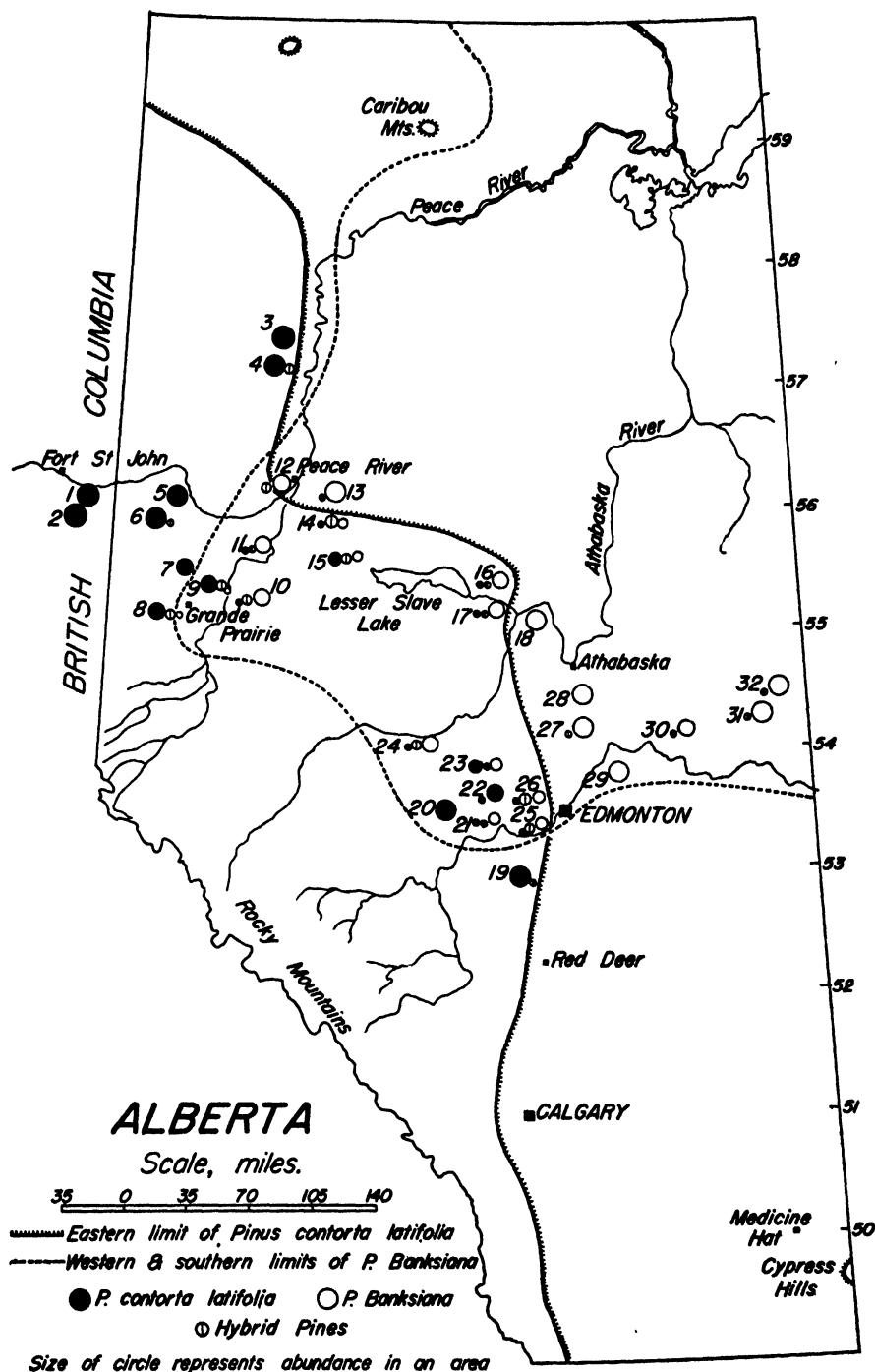
This paper deals with the occurrence in Alberta of the two closely related species, *Pinus contorta* Loudon var. *latifolia* Engelm. (*P. Murrayana* Balf.) and *Pinus Banksiana* Lamb. (*P. divaricata* Du M. de C.), and also with field evidence of extensive hybridization between these species where their ranges overlap.

*Pinus contorta latifolia*, commonly known as lodgepole pine, has an eastward extension in Alberta as shown on the accompanying map (Fig. 1). The lines representing the occurrence of this species, both the general eastward range and the outliers associated with certain hills, follow closely those published by Halliday and Brown (6), except for the region between Edmonton and the Peace River area. In this region, the line has been drawn to conform to the results of the present study. *Pinus Banksiana*, commonly called Banksian or jack pine, extends westward and southward in Alberta as shown by the broken line (Fig. 1), this line being approximately that published by Halliday and Brown (6), except where altered in the Edmonton - Peace River region in accordance with data of the present investigation.

In the overlapping region, where both species grow, there are also found many trees with characters intermediate between those of the two species. The only published report of this situation appears to be in the form of a personal communication by the present writer to Dr. H. M. Raup (12) in which the writer stated his observations of 1941 as follows: "*Pinus Banksiana* seems to prevail at Smith and on the north shore of Lesser Slave Lake opposite Widewater and Canyon Creek. Between Lesser Slave Lake and Peace River, *P. Banksiana* and *P. Murrayana* were observed. At Peace River town both species grow intermixed, with jack pine predominating. Trees showing intermediate characters were rather common in the Slave Lake - Peace River region". During the past four years the writer has made brief studies on the pines in numerous areas throughout a considerable portion of the region. These studies, presented in this paper, are to be regarded as only a preliminary

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account of the leading pine types and their occurrence in the region. They serve also to suggest various ecological, phytogeographical, and genetical problems worthy of critical investigation.

### Contrasting Features of the Species

The following discussion is based on: (a) descriptions in standard manuals, including those of Britton (3), Dallimore and Jackson (4), Elwes and Henry (5), Hough (8), Morton and Lewis (10), Rehder (13), Rydberg (14), Sargent (15), Sudworth (18); (b) critical evaluation of characters in *Pinus* by Shaw (16); (c) recent studies by the writer.

#### TREE HABIT

Lodgepole pine in Alberta generally grows in dense stands where it develops a tall, straight, clean trunk. Open stands of the pine are not uncommon in the region studied. The trees of these stands have broad crowns and usually retain their branches near the trunk base. The ends of the branches show a pronounced tendency to curve upward, contrasting with the generally drooping branches of wide-spreading jack pine.

Jack pine, in most of the areas studied, exhibits the wide crown and rather scrubby growth commonly regarded as characteristic of this species. But, as has been reported for various other parts of Canada, so in Alberta, dense stands of this pine have trees with tall, slender, clean trunks and small crowns.

Therefore, while general tree habit cannot be relied upon as a diagnostic feature, the semi-erect branch form of the lodgepole contrasts with the drooping branches of the jack pine, at least in older trees with wide crowns. This difference in branching habit has been observed in lodgepole and jack pine growing side by side, thus indicating that it is an inherent rather than a habitat response. Pines with various of the intermediate characters described in this paper usually show also an intermediate branch habit.

#### BARK OF THE TRUNK

According to most authorities, the outer bark of the lodgepole pine is darker in color, and characterized by finer and more uniform scales, than that of the jack pine. Whereas the lodgepole bark forms small, loosely appressed scales, the jack pine splits into irregular, scaly ridges and furrows. While these differences appear to be fairly constant for the two species in our region, definite conclusions as to bark characters of the intermediate pine types are not indicated but might be reached through an extensive investigation.

#### FOLIAGE

Lodgepole pine is described as having considerably longer, less twisted and less divergent needles than jack pine, and arranged in more dense clusters toward the ends of the twigs. In our experience, these are fairly good

diagnostic features for the species. While we have made no critical studies on needle length, intermediates between the species appear generally to have the jack pine type of foliage.

#### LEAF ANATOMY

Harlow (7) employs shape of leaf epidermal cells to distinguish these two species. The epidermal cells of lodgepole pine are said to appear nearly square, those of jack pine rectangular in transverse section. In our experience this is wholly unreliable as a distinguishing feature. Examination of numerous collections has failed to reveal any constant difference in the epidermal cells of the two species. This anatomical character seems therefore to have no value for the diagnosis of intermediate forms.

#### STAMINATE CONES AND POLLEN CHARACTERS

The pollen sacs of lodgepole pine are said to be orange or orange-red, those of jack pine yellow in color. Since most of our field studies were done after the staminate cones had matured, we are unable to express an opinion as to the reliability of this feature; nor have we been able to use this as a criterion for determining affinities of intermediate pine types.

We have evidence that where the two pine species grow together, jack pine sheds its pollen a few days in advance of lodgepole. For instance, at Heatherdown (Area No. 26), on June 2, 1948, typical jack pine was shedding pollen, while the pollen cones of lodgepole were still greenish. A tree of an intermediate type (similar to that shown in Fig. 9) was just beginning to shed pollen, thus indicating a closer affinity with jack pine than with lodgepole. Pollen of this intermediate pine type was found to be about 50% normal in appearance and 50% distorted and shrunken, while pollen of typical jack and lodgepole trees, collected at the same time, showed practically no distortion of grains.

#### SEED CONES

##### *Position of Cones on Stem*

Certain authorities, including Sargent (15), Elwes and Henry (5), place *Pinus contorta* and *P. Banksiana* in different Sections of the genus, on the understanding that *P. contorta* bears its cones subterminally on uninodal shoots, while *P. Banksiana* has its cones lateral in position on multinodal shoots. Shaw (16) established that these characters should not be employed for grouping species of *Pinus*, "for Pines are not sharply divided into multinodal and uninodal species, and no exact segregation of them based on this difference is possible". Actually Shaw describes both *P. contorta* and *P. Banksiana* as multinodal, although he emphasizes that "the multinodal shoot is never invariable in a species, but is rare, common or prevalent". Unfortunately, cone position was used later by Sterrett (17) to distinguish these two species. Still more unfortunate is Sterrett's statement that jack pine has the cones subterminal and lodgepole pine has the cones lateral, which reverses the distinction emphasized by the earlier taxonomic treatments. Sterrett's statement led us to make a few counts on local plantations of typical lodgepole

and jack pine. The result for lodgepole was seven lateral to eight subterminal; for jack pine, 36 lateral to one subterminal. These results are in accord with Shaw's conclusions. It is probable, therefore, that data on cone position have little or no value for an interpretation of our intermediate forms of pine.

### *Cone Characters*

Features of the mature seed cones are now generally considered to be of prime importance in distinguishing lodgepole and jack pines. Most authorities emphasize these differences somewhat as follows. The mature cone of lodgepole pine is spreading or reflexed, ovoid or conical in shape, the scales conspicuously umbonate, each scale armed with a minute recurved prickle (Fig. 2). The mature cone of jack pine is erect (directed toward the apex of the shoot), strongly incurved or slightly spreading, the scales variously thickened (the outer and lower, large, mammiform) and unarmed (Fig. 3). This characterization is supported by the present study.

Actually, a number of the manuals do not draw the foregoing sharp distinctions between the two species, and probably for the following reasons. (a) Numerous intermediate forms do occur, such as those of the overlapping region dealt with in this paper. (b) Quite outside the overlapping region, there are known to be forms of these pines that do not conform to either of the descriptions given above. The most baffling type of pine encountered in this investigation has spreading (horizontal), straight, smooth cones (Fig. 4). This form of pine is known to occur far eastward in Canada. Mr. A. J. Breitung (letter of Aug. 17, 1947) informs me that he has cones of this type from Nipawin, Sask., and Mr. J. S. Rowe (letter of Jan. 22, 1948) has supplied information, including photographs, of the same type growing in Riding Mountain Park, Man. For our present purpose this pine type is regarded as a form of *Pinus Banksiana*, as also is the type shown in Fig. 5, which is intermediate in cone characters between the former and typical jack pine.

Our somewhat arbitrary decision to regard this type (Fig. 4) as a form of jack pine, rather than an intermediate form, is based mainly on the following considerations: (a) its occurrence far eastward beyond the present range of lodgepole pine; (b) considerable evidence that it is more prevalent in stands composed chiefly of jack pine than in mixed stands of jack and lodgepole pines; (c) its branching habit and needle characters are, in our experience, those of typical jack pine. Admittedly, there are good reasons for adopting the alternative view, viz. that this type of pine is a lodgepole-jack hybrid. This aspect of the subject is discussed more fully later in the paper.

In spite of certain complications, such as those just indicated, cone characters are judged to be the best readily available diagnostic feature for these pines. The following presentation of intermediates (hybrids) between the two species is based, in large part, on characters of the mature seed cones.

It should be noted that the mature cones show remarkably little variation in any particular tree, regardless of the type.

PLATE I

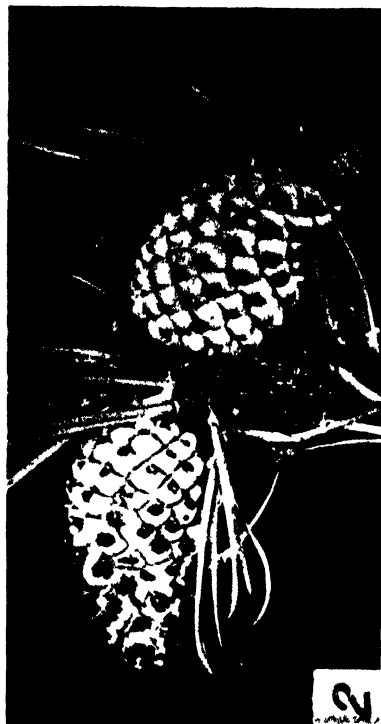


FIG. 2. *Pinus contorta latifolia*; typical cones; approx.  $\times 0.8$ .



FIG. 3. *Pinus Banksiana*; typical cones; approx.  $\times 0.8$ .



FIG. 4. *P. Banksiana*; straight, horizontal cone; approx.  $\times 0.8$ .



FIG. 5. *P. Banksiana*; semi-erect cones; approx.  $\times 0.8$ .



PLATE II



FIGS 6 to 9 *P. contorta latifolia*  $\times$  *P. Banksiana*. Fig 6, reflexed, smooth, Fig 7, horizontal, smooth, with recurved tip, Fig 8, erect, incurved, spiny, Fig 9, erect form, weak but persistent spines (All figures are approximately  $\times 0.8$ )

## Hybrids in the Transition Region

Where the ranges of lodgepole and jack pine overlap, the two species and the many intermediate forms occur in varying proportions from place to place. Of a large number of stands examined, 32 are described below and shown on the outline map (Fig. 1). The circles on the map give only an approximate idea of the relative abundance of the species and hybrids. The largest circles represent a very high frequency; the smallest circles indicate a rare occurrence. The considerable space occupied by the circles on the map is not to be taken as a measure of pine forest in the region. Actually, pine stands are relatively uncommon in most parts of the region covered by this investigation and comprise only a small fraction of the total forest cover.

The main types of intermediates (hybrids) are as follows:

(a) Illustrated in Fig. 6; cone reflexed and smooth; like typical lodgepole in shape, resembling jack pine in lacking prickles; generally associated with the jack pine type of foliage; a rather rare type.

(b) Shown in Fig. 7; cone nearly horizontal, with a recurved tip, smooth or with weak prickles; resembles the form of jack pine (Fig. 4) with straight, horizontal cones and may conceivably be more closely related to that form than to typical lodgepole; a fairly common type.

(c) Shown in Fig. 8; cone erect, markedly incurved and with strong prickles; like lodgepole in being prickly but close to typical jack pine in shape; sometimes on trees with an upright branch habit and long needles crowded near the branch tips, evidence of lodgepole ancestry; a rather common type.

(d) Illustrated in Fig. 9; cone erect, somewhat incurved, and with weak prickles; usually associated with the jack pine branching habit and type of foliage; common in most parts of the transition region.

(e) Not illustrated; cone semi-erect and prickly; found occasionally.

(f) Not illustrated; cone semi-erect, tip recurved; probably close to jack pine; fairly common.

(g) Not illustrated; cone reflexed, weak spiny; evidently close to lodgepole; apparently rare.

(h) Not illustrated; exceptionally large cone, semi-erect, smooth; on trees with branching and foliage approaching the lodgepole type. Mention may be made here that trees with enormous, erect, incurved, smooth cones, and vegetative characters of the jack pine type have been seen; these appear to be typical jack pine in every way, except for the unusually large cones.

It is evident that almost every conceivable combination of lodgepole and jack pine cone characters has been found in the region. Trees with these intermediate characters are most abundant, (a) in the central parts of the overlapping region, (b) in stands where both lodgepole and jack pine occur. Conversely, trees with intermediate characters are relatively rare, or absent, (a) near the range limit of each species, and beyond that limit, (b) in stands

where only one of the species has been found. These observations are believed to lend strong support to the view that those pines with intermediate characters are indeed hybrids with lodgepole and jack pine ancestry.

### Data for Pine Stands

- No. 1. Dawson Creek – Fort St. John, B.C. An area with considerable variation in soil and topography. Lodgepole only seen; trees mostly tall and straight, a few with somewhat spreading habit.
- No. 2. Progress, B.C., about 25 mi. west of Dawson Creek. Typical lodgepole pine, growing with poplars and white spruce.
- No. 3. Hawk Hills, Alta. Lodgepole only observed; intermixed with poplars and spruce.
- No. 4. North of Meikle River. Chiefly lodgepole. A few intermediates (Figs. 8, 9).
- No. 5. Blueberry Mountain. Variable soil and topography. Lodgepole only seen.
- No. 6. About 25 mi. west of Spirit River. Nearly all typical lodgepole. Very few intermediates (Fig. 9).
- No. 7. Saddle (Burnt) Hills. Lodgepole only seen.
- No. 8. Grande Prairie. About four miles southwest of Grande Prairie, near the Wapiti River; a sand-hill area. Lodgepole abundant, most with reflexed, some with horizontal, cones. Intermediates (Figs. 8, 9) scattered throughout. Jack pine occasional and localized.
- No. 9. Kleskun Hill, about 12 mi. northeast of Grande Prairie. Hilly, "badland" area; small groves and isolated trees. Lodgepole pine abundant. Intermediates (Fig. 9) occasional. Jack pine very rare; one tree seen.
- No. 10. Smoky River, east of Grande Prairie. Rough topography. Pines on slopes and flat areas in valley. Lodgepole occasional. Intermediates, various types (chiefly Fig. 9), scattered. Jack pine, typical form, abundant. Jack pine (Fig. 4) rare.
- No. 11. Southeast of Codesa. Level, sandy area. Typical lodgepole, rare. Intermediates rare. Jack pine, typical form, abundant. Jack pine (Fig. 4) scattered.
- No. 12. Peace River (town). Sandy and gravelly flats of river valley. Trees of various growth forms, mainly with wide crowns. Intermediates, various forms, including those illustrated (Figs. 6 to 9), comprise somewhat over one-quarter of the stand. Typical jack pine, about one-half of the trees. Other forms of jack pine, about one-fifth of the stand.
- No. 13. Harmon Valley, 20 mi. east of Peace River. Sandy area, rolling topography. Intermediates (Figs. 8, 9) occasional. Jack pine, typical, abundant; (Fig. 4) occasional.

- No. 14. McLennan-Nampa. Mostly heavy soil and flat topography. Lodgepole and jack pine frequent. Intermediates, including types illustrated (Figs. 7, 8, 9), more abundant than either of the species. Growing with aspen, balsam poplar, and white spruce.
- No. 15. Triangle. Mainly a low, flat area. Lodgepole, intermediates of various kinds, and jack pine are all well represented. Pines tall and slender, forming pure stands locally, otherwise with poplars and white spruce.
- No. 16. Lesser Slave Lake, at east end. Sand dune area. Lodgepole rare. Intermediates rare. Jack pine, typical, abundant; (Fig. 4) occasional.
- No. 17. Eight miles southeast of Slave Lake (town). Low sandy area, bordering peat bog. Lodgepole, typical, occasional to scattered. Intermediates occasional. Jack pine, typical, scattered; (Fig. 4) occasional.
- No. 18. Smith. Sand hills. Jack pine, typical, abundant; (Fig. 4) occasional.
- No. 19. Battle Lake, west of Pigeon Lake. Fairly low area with variable soil, some heavy. Lodgepole, typical, abundant. Intermediates, similar to Fig. 9, but less incurved and with stronger spines, rare.
- No. 20. Lobstick. Low flat area. Dense stand of pine and black spruce. Typical lodgepole, abundant.
- No. 21. Wabamun Lake, near Seba. A fairly flat, sandy area. Pines mainly in open stands. Lodgepole occasional. Intermediates occasional. Jack pine, typical, common; (Fig. 4) rare; (Fig. 5) occasional.
- No. 22. Evansburg. Sandy to heavy soil. Lodgepole abundant. Intermediates (Fig. 8), rare.
- No. 23. Sangudo. Somewhat sandy area. Lodgepole frequent. Intermediates occasional. Jack pine frequent.
- No. 24. Whitecourt. Sandy region; flat to rolling topography. Lodgepole occasional. Intermediates scattered, including those shown in Figs. 6 to 9 and other forms, e.g. reflexed, weak spiny; semi-erect and spiny. Jack pine, typical, frequent; (Fig. 4) rare.
- No. 25. Graminia, about 20 mi. southwest of Edmonton. Sand-hill area. Lodgepole rare. Intermediates of many kinds, including types shown in Figs. 6 to 9, scattered. Jack pine, typical, frequent; (Fig. 4) rare.
- No. 26. Heatherdown, about 25 mi. northwest of Edmonton. Gravelly, hilly area. Lodgepole rare. Intermediates (Figs. 7 to 9) and other kinds, scattered. Jack pine, typical, scattered; (Fig. 4) occasional; (Fig. 5) rare.
- No. 27. Nestow. Sand-hill area. Intermediates (Figs. 6, 7) rare. Jack pine, typical, abundant; (Fig. 4) occasional; (Fig. 5) rare.

- No. 28. Rochester. Sand-hills. Jack pine, typical, frequent; (Fig. 4) scattered; (Fig. 5) occasional; also a few trees with exceptionally large cones, otherwise typical jack pine.
- No. 29. Bruderheim. Sand-hill area. Jack pine, typical, abundant; (Fig. 4) occasional (1 to 2% of the latter type).
- No. 30. Bellis. Somewhat sandy, flat to gently rolling, area. Intermediates occasional; various types, including those shown in Figs. 6, 7. Jack pine, typical, frequent; (Fig. 4) occasional; (Fig. 5) occasional to scattered.
- No. 31. St. Paul-Bonnyville. Sandy, flat area. Intermediates (Fig. 7) rare. Jack pine, typical, abundant; (Fig. 4) occasional; (Fig. 5) occasional.
- No. 32. Beaver River. Sandy, rolling area; near river valley. Intermediates (Fig. 7) rare. Jack pine, typical, abundant.

### Discussion

#### RANGE LIMITS OF THE SPECIES

On the map (Fig. 1) the heavy lines are believed to represent fairly accurately the range limits of lodgepole and jack pine as understood by Halliday and Brown (6) and modified locally by the present writer. Further observations will doubtless necessitate certain changes in these lines, especially in the relatively unknown areas between Whitecourt and Grande Prairie, northeast of Lesser Slave Lake, and throughout the more northern region.

#### ECOLOGICAL CONSIDERATIONS

Our field data point to the conclusion that lodgepole is more prevalent than jack pine on the heavier soils, while jack pine is much more abundant on sandy areas. Striking exceptions to the rule do occur, for instance, the extensive growth of lodgepole pine on sand hills southwest of Grande Prairie. It is probably significant that this area is virtually in the lodgepole pine belt and close to the western limit of jack pine. Worthy of note too is the fact that lodgepole and jack pine are occasionally found growing side by side even on sandy areas. Both species appear to thrive on gravelly or stony knolls and river flats. Conclusions concerning the relation of the intermediate forms to site are not clearly indicated. It is obvious that an adequate understanding of adaptational aspects of these pine types must await critical investigation.

Lodgepole pine is commonly associated with other tree species, principally *Picea glauca* (Moench) Voss, *Populus tremuloides* Michx., and *P. Tacamahacca* Miller, these occurring in varying proportions and often as dense stands of tall, straight trees. Jack pine usually forms open stands, with relatively few trees of the associated species, *Populus tremuloides* and *Betula papyrifera* Marshall, these being small and scrubby. Where jack pine occurs on the heavier soils, it is generally associated with white spruce, poplars, lodgepole pine, and hybrid pines, all with a slender habit and in close stands.

## PHYTOGEOGRAPHICAL ASPECTS

It is probable that lodgepole and jack pine have migrated into the overlapping zone, from west and east respectively, during a rather recent period. On the other hand, the view that both species have been long established in the region finds some support in their distribution, which is at once extensive and discontinuous. It is possible too that at an earlier period the pines had an even greater coverage there than at the present time. Of interest in this connection is Raup's interpretation (11) of certain pine-spruce relationships in the country between Athabaska and Great Slave Lakes. Raup suggests that the earliest postglacial forests were of white spruce arranged in an open, parklike association and that, with the warming of the climate and decrease of ground frost, the less tolerant pine could enter and compete successfully in the spruce stands on sandy areas. With further amelioration of climate and improvement in the moisture and humus conditions, there was a tendency for spruce to suppress the pine and again to dominate these areas. Changes along these lines may have occurred in the sand-hill parts of our region, though nearly all of these appear to be held at the pine stage, probably as a result of frequent burning. Similar changes may have taken place on flatter areas and on heavier soils, leading to the mixed spruce-pine-poplar forest of the present time. Involved here are *Populus tremuloides* and *P. Tacamahacca*. Like the pines, the poplars tend to be succeeded by white spruce, but are favored by occasional burning. There is a distinct possibility, therefore, that the pine species at one time dominated larger areas in the region.

Of interest here is Hultén's view (9) that the "Linnaean species" of the boreal flora originated in the last great interglacial or earlier and that the present areas of boreal biota are, on the whole, reductions from the areas that had developed already during interglacial times. Accordingly, lodgepole and jack pine may be assumed to have occurred as distinct species and with overlapping ranges during interglacial times. If so, they must have formed hybrids even as they do now. Moreover, during a long interglacial, certain of the transition forms were probably segregated in response to special conditions or by geographical barriers or otherwise. The possibility of such "hybrid" forms surviving glaciation in refugia and later returning to the region is of interest in connection with the aberrant jack pine types (Figs. 4, 5). While this suggestion may be highly speculative, it should not be dismissed until exploration has established the absence of aberrant pine types from those areas southeastward from Manitoba that were presumably supplied with pines from the Wisconsin refugium.

## ASPECTS OF HYBRIDISM

The criteria for hybridism employed in this study are essentially those approved by Allan (1) for wild species hybrids. These criteria may be stated briefly as follows: the occurrence of an assemblage of forms more or less intermediate between the putative parent species, these forms comprising a

graduated series linking one species with the other and generally found in proximity with, but rarely apart from, the parent species.

The occurrence of pine hybrids well beyond the present eastern limit of lodgepole pine requires an explanation. This is at hand if the hypothesis of a more easterly range for this species in earlier postglacial time is accepted. An alternative hypothesis is in terms of the eastward migration of certain hybrids well adapted for the prevailing conditions. Of interest in this connection is the phenomenon of introgressive hybridization that has been demonstrated for several groups of plants through the work of Anderson (2) and others. It may well be that we have introgression of lodgepole into jack pine, but this has yet to be established by biometric analysis.

As already suggested the aberrant jack pine types (Figs. 4, 5) may actually be hybrids derived from a postglacial union of lodgepole and typical jack pine. The occurrence of at least one of these types (Fig. 4) as far east as Manitoba seems to make considerable demands upon a migration hypothesis, especially if this assumes lodgepole pine to have extended no farther east than its present range. An investigation of the pines throughout the region using the techniques employed in introgression studies would doubtless clarify our understanding of the whole situation.

### Acknowledgments

This investigation was carried out in connection with general botanical field work, supported during 1947 and 1948 by financial assistance from Science Service, Department of Agriculture, Ottawa, and for which grateful acknowledgment is made. Several colleagues, forest rangers, and others have helped in various ways. Special thanks is extended to Messrs. J. S. Rowe, R. L. Airth, F. C. Marfleet, and D. A. Fraser for valuable assistance in the field.

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## YIELD AND PROTEIN CONTENT OF WHEAT AND BARLEY

### I. INTERRELATION OF YIELD AND PROTEIN CONTENT OF RANDOM SELECTIONS FROM SINGLE CROSSES<sup>1</sup>

By M. N. GRANT<sup>2</sup> AND A. G. MCCALLA<sup>3</sup>

#### Abstract

Yield and protein content of 100 random selections from single crosses of wheat and barley were determined for three years. Results of each test showed highly significant differences in yield and protein content of selections. There was a highly significant negative correlation between yield and protein content in each test, the values for  $r_{py}$  varying from  $-.483$  to  $-.806$ . In general, the relationship was improved when mean values from several tests were correlated. The behavior of individual selections under a wide range of environmental conditions was relatively constant even though wide differences occurred in both yield and protein content of individual selections.

#### Introduction

Data from many sources suggest that there is a significant negative association between yield and protein content of wheat and barley (1, 3), and it seems altogether likely that part of this association is, in some manner, genetically controlled. The association has great practical importance if it is consistent and close. The plant breeder will find it difficult to develop bread wheats that give maximum yields and yet have satisfactorily high protein content. On the other hand, varieties of malting barley and wheat for pastry flour will likely have high yield associated with reasonably low protein content, a condition that is desired.

Some of the early literature pertinent to this problem was reviewed by Neatby and McCalla (3) and extensive new data were presented. Since then, the most important contribution has been made by Berg (1) working with winter and spring wheats in Sweden. He used selections from hybrid populations, but these differed radically from those used in the present study inasmuch as rigid selection had been practised in earlier generations to eliminate strains that were not winter hardy, were weak, low in yield, or poor in quality. Selection for yield and quality certainly had the effect of reducing the range of yield and protein content in the selections studied. Nevertheless, Berg obtained results agreeing well with those published earlier. Correlation coefficients of  $-.578$  and  $-.607$  were obtained for winter and spring wheats. While he found that it is possible to combine higher yield with higher protein content, the extent to which such combination can be secured seems limited.

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While the study by Neatby and McCalla (3) was under way, it appeared that a true measure of the correlation between yield and protein content, as it pertained to breeding, could be obtained only by working with random selections from hybrid populations of wheat and barley. This paper reports the results obtained over a period of three years' study with such material.

### Material and Methods

In selecting parental material for the production program, chief attention was directed toward the yielding ability and protein content of each potential parent. Obviously, greater ranges could be expected by crossing varieties that most clearly exhibited extremes in the characteristics to be studied. Although other factors were not disregarded, one of the parents used was high in yield but low in protein, while the other was low in yield but high in protein.

#### *Barley*

The barley parents selected were Trebi and Peatland. Trebi is consistently a very high yielder, but is one of the varieties lowest in protein. Peatland is a low yielder, but has usually shown very high protein content under Alberta conditions. The cross was made in 1936. The seed from the cross was grown in bulk plots until 1939 when 100 random selections were made of plants carrying  $F_4$  seed. The selected material was planted in head rows in 1940, and in 1941 the increased seed was used in a quadruplicated yield test at Edmonton. Moisture conditions were very poor and the stand was uneven, with late selections suffering much more than early ones. Useful results were scarcely expected from this test.

Owing to labor shortages arising from the war further work had to be postponed until 1944. In 1944 and again in 1945, 100 random selections were grown at Edmonton, while in 1946, 50 of these 100 samples were grown on black loam soil at Edmonton and also at Fallis, located 50 mi. west of Edmonton, on a relatively infertile podsolic loam soil.

#### *Wheat*

The parents used in the wheat study were Bunyip and Dicklow. Both are soft white wheats: Bunyip is characterized by low yield and high protein, while Dicklow has consistently been a very high yielder but has had the lowest protein content of any variety grown under local conditions. The cross was made in 1936 and the seed grown in bulk plots until 1940, when a random selection of heads from  $F_4$  plants was made. These were increased in 1941. One hundred of these selections were used in yield trials at Edmonton and Fallis in 1944 and 1945, while 50 of the 100 were used at each place in 1946.

#### *Design and Production*

Each field test in 1944 and 1945 was in the form of a  $10 \times 10$  simple lattice (2) with four replicates. Standard rod-row plots with three rows and 9 in. spacing were used. Seeding of barley was at the rate of 2 bu. per acre, and of wheat at  $1\frac{1}{2}$  bu. No fertilizer was used. Only the center row of each plot

was harvested. Each replicate was threshed and weighed separately but, except in one year, protein determinations were made on composite samples.

In 1946 the 50 selections were handled in the same manner except that the design used was a simple randomized block.

### *Weather Conditions*

**1941.** Weather conditions were decidedly adverse in 1941. The season was dry, and later selections suffered more from drought than did earlier ones. It was anticipated that the marked differential effect on early and late selections might well obscure any relationship between yield and protein content.

**1944.** Growing conditions were excellent throughout the season of 1944. There was 11 in. of rainfall at Edmonton in the period May, June, and July. Crops were very heavy, and no serious setback should have been suffered by any selection of barley or wheat at Edmonton. Growing conditions at Fallis were also very good.

**1945.** The growing season in 1945 was dry. Only 4.7 in. of rain fell during the period May, June, and July at Edmonton. In spite of this, yields were good, and crops developed normally at both Edmonton and Fallis.

**1946.** Growing conditions at both Edmonton and Fallis were excellent in 1946. Lodging occurred with many selections, particularly at Edmonton, but careful handling largely eliminated errors that might have occurred in yields.

### *Protein Determination*

All protein results were determined using the Kjeldahl method with mercuric oxide as catalyst. Protein in wheat is reported as  $N \times 5.7$  and in barley as  $N \times 6.25$ . All results are expressed on the basis of 13.5% moisture.

## **Results**

### *Barley*

The results of all barley experiments are summarized in Tables I and II, and in Figs. 1 and 2.

TABLE I  
ANALYSIS OF VARIANCE, REPRESENTATIVE BARLEY DATA

Variance due to:	Edmonton, 1945				Yield, 1946		
	D.f.	Mean squares			D.f.	Mean squares	
		Yield	Protein	Growth period		Edmonton	Fallis
Selections	99	514.06**	3.80**	146.5**	49	274.41**	336.83**
Replicates	3	4851.70**	4.83**	188.0**	3	243.07	4.62
Error	297	57.73	0.33	2.7	147	135.00	82.02

\*\* Exceeds the 1% point.

The 1941 results can be dismissed with the brief comment that, despite the very adverse growing conditions, a negative correlation coefficient of

TABLE II  
CORRELATION AND REGRESSION RESULTS FROM BARLEY DATA

Station and year	D.f.	Statistic		
		$r_{PY}$	$b_{PY}, \%/\text{bu.}$	$b_{YP}, \text{bu.}/\%$
Edmonton 1941	98	-.300**	-0.037	-2.8
1944	98	-.533**	-0.044	-6.5
1945	98	-.761**	-0.066	-8.8
1946	48	-.483**	-0.050	-4.7
Mean 1944-46	48	-.793**	-0.085	-7.4
Fallis 1946	48	-.762**	-0.091	-6.3

\*\* Significant beyond the 1% point.

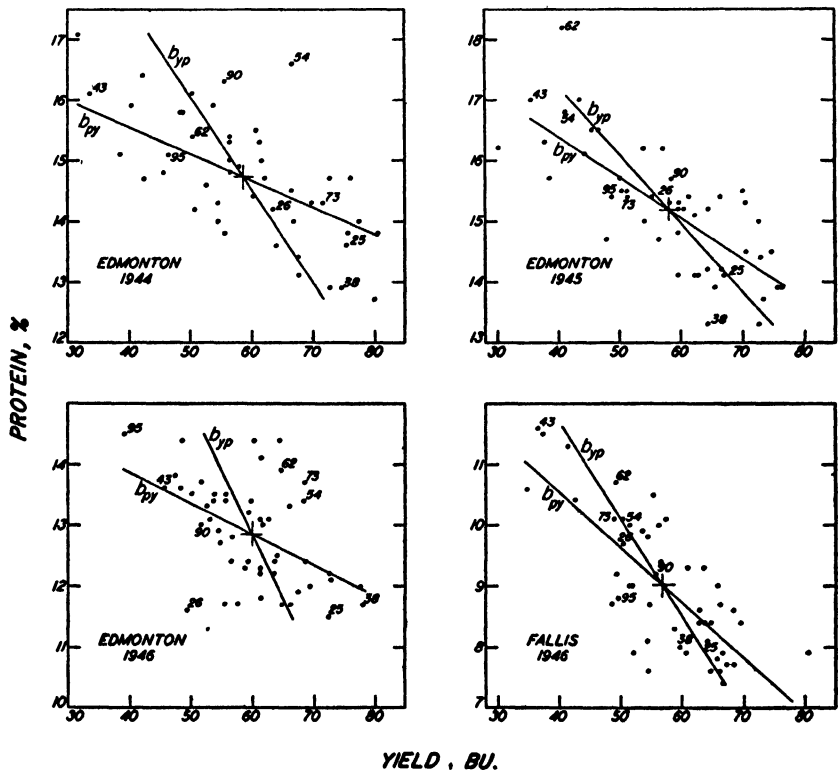


FIG. 1. Correlation surfaces for individual tests of barley showing the interrelation between yield and protein content.

— .300\*\* was obtained. The regression coefficient  $b_{py}$  was — 0.037% per bushel, which is somewhat lower than most results obtained by other investigators. This is to be expected with the comparatively low correlation coefficient obtained.

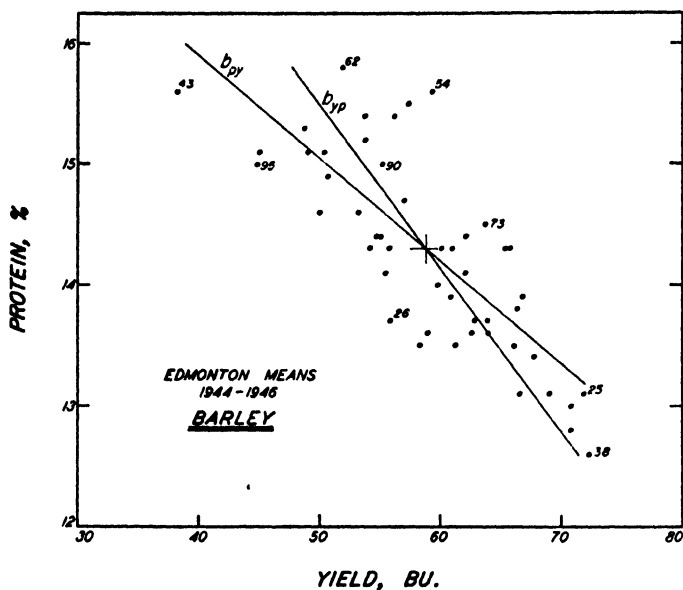


FIG. 2. Correlation surface for the mean values from three barley tests, showing the interrelation of yield and protein content.

Representative results of analysis of variance are given in Table I. Highly significant variation in both yield and protein content of individual strains occurred in all years. Time of maturity also varied widely in 1944 and 1945. No maturity notes were taken in 1946. The high variation in growth period was negatively associated with yield in 1944 and positively associated with yield in 1945. This difference had some effect on the numerical value of the correlation between yield and protein content but did not alter the nature or generality of the conclusions regarding this relationship. For this reason it is not considered necessary to discuss it further, except to note that it might be of some importance if practical applications of the yield-protein relationship were to be developed.

The correlation and regression coefficients are presented in Table II, while the correlation surfaces are plotted in Figs. 1 and 2. The highly significant correlation shown in every test leaves no doubt that the negative relationship between yield and protein content is the normal condition to be expected. In general, the coefficients are higher than most of those presented by other investigators and reviewed in the paper by Neatby and McCalla (3). The three-year means for results obtained at Edmonton should have the effect of

\*\* Throughout the paper, a highly significant correlation is marked with two asterisks, and a significant coefficient with one.

largely eliminating environmental factors influencing the correlation. It seems definite that at least part of the association of low protein and high yield is controlled genetically, although the actual internal effects resulting in the association are almost certainly physiological in nature. This thesis could be developed but it seems advisable to leave such discussion to a later paper.

Two kinds of regression coefficients are given in Table II. Investigators interested in the general association of yield and protein content would probably plot the data as is done in Figs. 1 and 2 with yield as the independent variable. Thus one would think of variations in yield affecting the level of protein content. Perhaps, however, a much more valuable use can be made of data such as these. If yields of selections could be predicted in early stages of a breeding program it would save the plant breeder a great deal of expense and time. A protein test can be made much more cheaply and with much less material than can a yield test. It is of interest, therefore, to calculate  $b_{yp}$ , since this is the coefficient that must be used if prediction of yield is to be made.

The results in Table II show that in general a reduction of about  $-0.05\%$  or more in protein content may be expected for every bushel increase in yield. The  $b_{py}$  values are usually higher with material grown on relatively infertile soils, such as occur at Fallis (3). If nitrogen supply is a limiting factor in plant development, obviously an increase in yield will mean that the nitrogen available must be distributed over more plant material, and the percentage will be lower. This is what occurs at Fallis. Despite this general change in physiological balance within the plant, the selections tend to occur in the same position on all correlation surfaces, a fact illustrated by the "tagged" values in Figs. 1 and 2.

The results for barley, therefore, substantiate earlier results. The correlation coefficients tend to be higher than in most studies, but this was expected since selection for other characters had not been carried out before the correlation studies were begun.

### *Wheat*

The results for the wheat studies are presented in Tables III and IV and Figs. 3, 4, and 5.

The analysis of variance results show much the same relationships as did those for barley. Selections varied widely in yield and time of maturity. In one year only were protein tests made on material from individual plots, but in this one test, highly significant differences in protein content were demonstrated. As with barley, it was found that the earliness or lateness of a selection was correlated with yield, but only at Edmonton in 1945 did this association affect the correlation between yield and protein. This effect was to increase the correlation coefficient when growth period variations were eliminated. The result given in Table IV for this test is, therefore, lower than that obtained for the partial correlation ( $-0.638$ ).

TABLE III  
ANALYSIS OF VARIANCE, REPRESENTATIVE WHEAT DATA

Variance due to:	Edmonton, 1945				Yield, 1946		
	D.f.	Mean squares			D.f.	Mean squares	
		Yield	Protein	Growth period		Edmonton	Fallis
Selections	99	156.36**	1.07**	25.2**	49	517.16**	302.48**
Replicates	3	1527.95**	5.59**	86.0**	3	268.19	205.40*
Error	297	61.62	0.26	5.5	147	100.76	73.83

\* Exceeds the 5% point.

\*\* Exceeds the 1% point.

TABLE IV  
CORRELATION AND REGRESSION RESULTS FROM WHEAT DATA

Station and year		D.f.	Statistic		
			$r_{py}$	$b_{py}$ , %/bu.	$b_{yp}$ , bu./%
Edmonton 1944		98	-.806**	-0.053	-12.3
	1945	98	-.527**	-0.048	-5.8
	1946	48	-.683**	-0.058	-8.1
	Mean	48	-.815**	-0.063	-10.6
Fallis 1944		98	-.666**	-0.091	-4.9
	1945	98	-.641**	-0.079	-5.2
	1946	48	-.762**	-0.063	-9.2
	Mean	48	-.781**	-0.073	-8.3
Edmonton mean yield		48	-.724**	-0.048	-10.9
Fallis mean protein					
Fallis mean yield		48	-.768**	-0.084	-7.1
Edmonton mean protein					
Edmonton-Fallis mean		48	-.872**	-0.075	-10.1

\*\* Significant beyond the 1% point.

The correlation coefficients are of the same order as those obtained with barley, although on the average they are slightly higher. Since the tests were located at two stations in each of the three years, it is possible to obtain correlation and regression coefficients for entirely independent samples. When the mean yields obtained at Edmonton are correlated with the mean protein values obtained at Fallis, or vice versa, the environmental influence should be entirely eliminated. Nevertheless, the correlation coefficients for such three-year mean values are high, and show that there is an inherent tendency for a selection to combine high protein and low yield (Fig. 5). The association tends to improve as wider means are used, since the highest value

for  $r_{py}$  was obtained using the mean yield and protein values for six independent tests over a three-year period at two stations. A comparison of the correlation surfaces in Figs. 3, 4, and 5 shows this closer relationship using means.

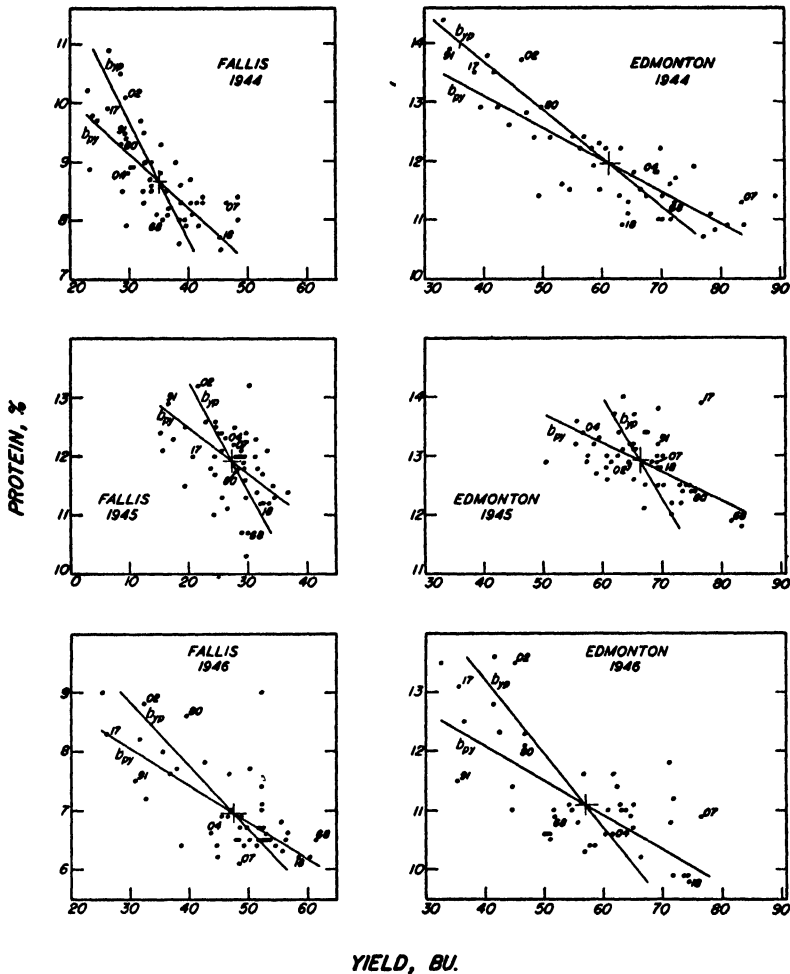


FIG. 3. Correlation surfaces for individual tests of wheat showing interrelations of yield and protein content.

The results in Fig. 4 are of interest in assessing the environmental influence on the yield-protein relationship. It is quite apparent that samples grown at Fallis, on low-nitrogen podsolic soil, are lower in protein and in yield than corresponding samples grown at Edmonton, on fertile black loam. The effect of the difference in environment has not changed the relative reaction of individual selections, however, as can be readily seen from the consistency with which the tagged selections retain their relative positions on the correlation surface. Thus within the limits of this study, environment affects the



general level of yield and protein content to an even greater extent ~~than does~~ variability among selections, but the relative effect on selections is more or less uniform. This general relationship is illustrated in a different way in Fig. 5.

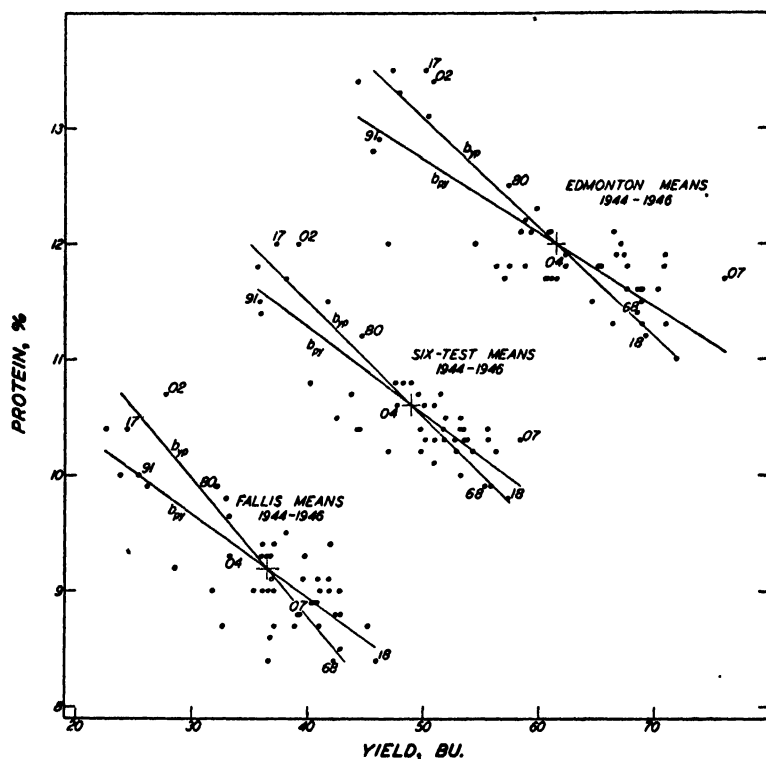


FIG. 4. Correlation surface for mean values from six wheat tests, showing the interrelation of yield and protein content.

### Discussion

The results presented in this paper confirm the general negative relationship between yield and protein content of wheat and barley. The generally higher correlation coefficients reflect the greater suitability of random selections from single crosses for this type of study as compared with varieties in ordinary tests or selections from single crosses where elimination based on other characters has already been carried out. Even the highest correlation coefficients, however, show that a considerable part of the variability in yield or protein is not necessarily associated with the variability in the other factor. Thus it should be possible to raise the protein content of high-yielding strains, but the writers agree with Berg (1) that the extent to which this can be done is probably not great.

The most practical use to which the relationship reported in this paper could be put would be in the early elimination of poor-yielding strains. If high protein means low yield in a large majority of cases, then the strains with

the highest protein content could be eliminated before any expensive yield tests were conducted. A correlation value of  $-.5$  is scarcely high enough to justify such elimination, but one of  $-.8$  would certainly justify it.

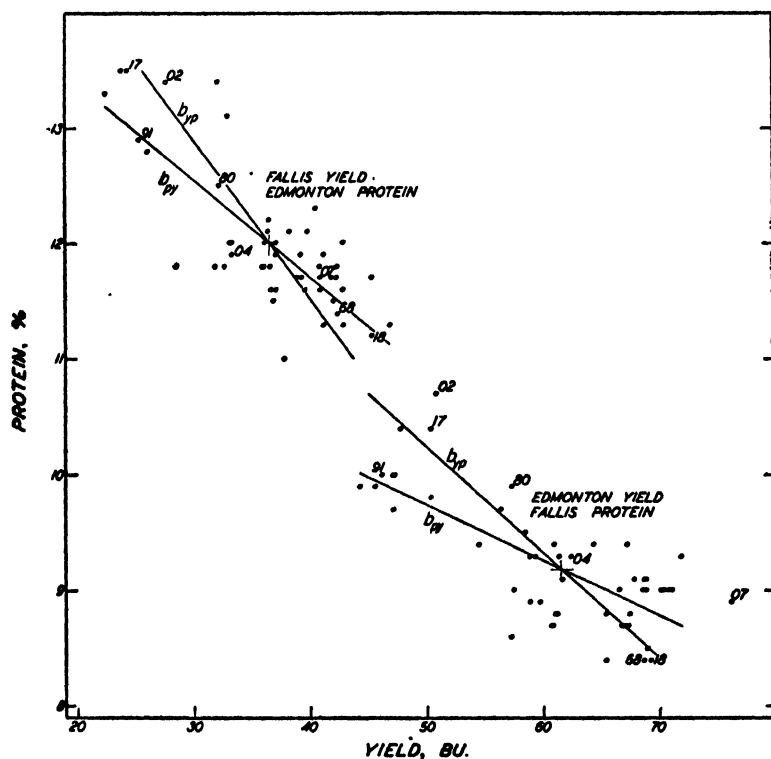


FIG. 5. Correlation surface for mean yield values obtained at one station plotted against mean protein values obtained at a second station. This should rule out environment as a factor affecting the inherited interrelation of yield and protein content.

Likewise a high value would be a reasonable guarantee that a large proportion of low protein selections would be high-yielding. Further discussion of the possibilities of using the relationship in this way is contained in the next paper of this series.

Reference has been made to the "inherent" tendency for yield and protein content to be negatively associated. It may be questioned whether there are specific genes for low protein or high protein, but it seems certain that the physiological factors that determine yield and protein content are genetically controlled. The high-yielding strains apparently nearly always absorb more total nitrogen than do low-yielding strains (1),\* but the percentage of protein in such strains is consistently lower. No attempt has yet been made to explain the physiological processes involved.

\* Calculations by P. F. Knowles on data obtained at Edmonton and Fallis substantiate Berg's results.

The suggestion outlined in the introduction to this paper can now be reliably confirmed. The results obtained in the present study make it clear that it is difficult to combine high yield and high protein content in wheat and barley. If this is a desirable combination, as with hard wheat, then breeding work is rendered difficult. If, however, high protein content is not a factor in determining high quality, as with wheat for pastry flour and barley for malt, the selecting of new varieties of these crops may be speeded up by taking advantage of the relationship studied in this project.

### Acknowledgments

The nature of the material used in this type of study necessitates that preliminary work be spread over a number of years. To Dr. K. W. Neatby, under whose supervision the crosses that provided selections for this study were made, and to the graduate students who assisted at various stages of the project, grateful acknowledgment is made.

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## STUDIES OF CANADIAN THELEPHORACEAE

IV. *CORTICIUM ANCEPS* IN NORTH AMERICA<sup>1</sup>BY H. S. JACKSON<sup>2</sup>

## Abstract

A parasitic basidiomycete, referred to *Corticium anceps* (Bres. & Syd.) Gregor, has been found to be common on a large number of hosts in the Timagami Forest Reserve, Ont. The same species is known to occur also in Quebec and in four of the northern United States. The fungus is transferred to the genus *Cerato-basidium*. The history of our knowledge of this parasite is reviewed, and a description and illustration of the fungus is provided as well as a discussion of the symptomology with illustrations of the effect on several hosts. A full list of the hosts on which the fungus has so far been found in North America, with the provinces and states in which collections have been made, is given in tabular form. The incompleteness of our knowledge with reference to host specialization, infection, and colonization of the host, and overwintering of the fungus is discussed and suggestions made for future work.

## Introduction

In connection with a general study of the mycological flora of the Timagami Forest Reserve in Northern Ontario, a parasitic basidiomycete which attacks a large number of hosts has been under observation for several years. The first collection was made in 1929 on *Cornus canadensis*. In 1931 and 1932 the same fungus was found on *Aralia nudicaulis* as well as on *Cornus* and was then identified as *Sclerotium deciduum* J. J. Davis. These collections were made in late August or September and were in the sterile condition. It was not until 1934 when collections were made between July 15th and August 1st that basidia were first noted. During that season the fungus was collected on 12 different hosts including *Pteridium aquilinum* var. *latiusculum*. Basidia were demonstrated on most of these hosts. The basidial fructification, as described below, is found only as a superficial film on the under side of the uninjured portions of the leaf in advance of the spreading necrotic lesions and is easily overlooked. The basidia are often absent or difficult to demonstrate in collections made later in the season but collections can be positively identified because of the presence and persistence of the characteristic infection cushions or appressorial pads, discussed later. In the fall of that year Dr. H. T. Güssow, who had visited Great Britain during the summer, called to my attention the preliminary papers by Mrs. Gregor (5, 6) dealing with a disease of the bracken fern in Scotland the causal fungus of which had been identified as *Tulasnella anceps* Bres. & Syd. and for which the combination *Corticium*

<sup>1</sup> Manuscript received June 15, 1949.

Contribution from the Department of Botany, University of Toronto, Toronto, Ont. This study was carried out, in part, with the assistance of a grant in aid of research furnished by the University of Toronto. Nos. I, II, and III of this series were published in *Can. J. Research*, C, 26 : 128-139 and 143-157, 1948; C, 27 : 147-156, 1949.

<sup>2</sup> The writer is indebted to Miss Charlotte E. Dill for the preparation of the drawings for the text figure, to Miss E. Ruth Dearden for assistance in the preparation of the manuscript, to Dr. J. H. Soper for checking the identification of most of the hosts, and to Dr. S. M. Pady for the privilege of examining recent collections made in Quebec.

*anceps* (Bres. & Syd.) Gregor was provided. Comparison of our material with Mrs. Gregor's description and with the type distribution of *Tulasnella anceps* (Sydow, Myc. germ. 858) left little doubt that our fungus should be referred to that species. In the meantime Mrs. Gregor's more detailed paper (7) on the same subject was published. Her extensive observations on the symptoms and progress of the disease, and on the morphology of the fungus as it occurs on bracken fern and the characteristics of the growth in culture, correspond so exactly with our own that there is little question that we had been concerned with the same species.

### History

*Tulasnella anceps* was originally described by Bresadola & Sydow (13, p. 490) on *Pteridium aquilinum*. No other host had been recorded for the fungus in Europe until Mrs. Gregor (7) reported its occurrence in nature on *Aspidium Filix-mas* in Scotland. In connection with her study of the disease on the bracken fern, however, Mrs. Gregor conducted a series of infection experiments in the course of which successful transfer of the fungus from bracken was obtained on eight other fern hosts, namely: *Aspidium Filix-mas*, *A. spinulosum*, *A. aculeatum* var. *lobatum*, *Asplenium Trichomanes*, *Polypodium vulgare*, *Blechnum spicant*, *Cystopteris fragilis*, and *Scolopendrium vulgare*.

Whether or not any name has ever been applied to the sterile condition of the fungus in Europe we have been unable to determine. In America, however, the late Dr. J. J. Davis (4) described *Sclerotium deciduum* from Wisconsin recording it on a number of hosts including both pteridophytes and angiosperms. Duplicates of several of these collections made by Dr. Davis were in the herbarium of the University of Toronto and an examination of these left no doubt that the fungus he described in the sterile condition and the one we have had under observation are the same. The first host listed by Davis in connection with the original description of *Sclerotium deciduum* was *Adiantum pedatum*. A duplicate in the herbarium of the University of Toronto, of a collection on that host made by Davis at Devil's Lake, Wis., Aug. 5, 1913, was examined and the basidia of *Corticium anceps* are present in beautiful condition. More recently, through the courtesy of Dr. E. M. Gilbert, all the collections of *Sclerotium deciduum* Davis which were then in the herbarium of the University of Wisconsin were examined for basidia. A total of 47 collections occurring on 17 hosts (see Table I) were included. Basidia were demonstrated on 17 of these collections occurring on nine hosts as follows: *Pteridium aquilinum* var. *latiusculum*, *Adiantum pedatum*, *Onoclea sensibilis*, *Pteritis pennsylvanica* (= *Onoclea struthiopteris*), *Aralia nudicaulis*, *Diervilla Lonicera*, *Hydrophyllum virginianum*, *Solidago serotina*, and *Lysimachia ciliata*. The other Wisconsin collections were made too late in the season to show the basidia or were made expressly to illustrate the sclerotial condition.

### Relationship

*Corticium anceps* as I have observed it and as described by Mrs. Gregor is quite certainly distinct from *Pellicularia filamentosa* (Pat.) Rogers (= *Corticium Solani* (Prill. & Del.) B. & G.). While both species are parasitic, *C. Solani* ordinarily attacks the underground parts of the host or the stem near the surface of the ground and the basidial fructification is ordinarily found at the base of the stem or petiole, only occasionally spreading out on the leaves of low-growing hosts. In *C. anceps*, on the contrary, the leaves or pinnae of the host are attacked directly. It has never been observed fruiting at the base of the stem. Then too the mycelium of *C. Solani* is of greater diameter than in *C. anceps* and does not develop the minute appressorial pads on the host or in culture which are so characteristic of the latter.

*Corticium anceps*, because of the character of the basidium and the habit of spore germination by repetition, belongs in the genus *Ceratobasidium* Rogers. This genus was erected by Rogers (11) to include a group of "delicate corticioid" forms, the older species of which had been included in *Corticium*, "whose holobasidia show the division into hypo- and epibasidia and whose spores" germinate by repetition. Whether or not one accepts *in toto*, Rogers' (10) interpretation of the basidial morphology in these groups, it is evident from a study of the forms included in *Ceratobasidium* that they represent a natural group of species. The species of *Ceratobasidium* possess characters which suggest relationship with the Heterobasidiomycetes, through the Tulasnellaceae on the one hand, and to the Homobasidiomycetes through *Pellicularia* (= *Botryobasidium* Donk.) on the other. In 1944, Martin (8) included *Ceratobasidium* in the Tulasnellaceae of the Tremellales. In a more recent paper Martin (9) has erected the family Ceratobasidiaceae to include the genus *Ceratobasidium*, placing it at the base of the Tremellales. He also suggests that *Pellicularia* and possibly related forms should sometime form the basis for a co-ordinate family at the base of the Homobasidiomycete series. In the opinion of the writer *Ceratobasidium* is too closely related to *Pellicularia*, as reviewed by Rogers (12), to justify such a wide separation in the classification of Basidiomycetes. It would seem more logical to include *Pellicularia* in the family Ceratobasidiaceae and place that family at the base of the Homobasidiomycete series. The two genera approach each other very closely, especially through the parasitic species.

### The Fungus

At present the writer is inclined to accept the genus *Ceratobasidium* as delimited by Rogers and to transfer to it the species under discussion:

***Ceratobasidium anceps*** (Bres. & Syd.) comb. nov.

*Tulasnella anceps* Bres. & Syd., Ann. Mycol. 8 : 490. 1910.

*Sclerotium deciduum* J. J. Davis, Trans. Wisc. Acad. Sci. 19 : 689. 1919.

*Corticium anceps* (Bres. & Syd.) Gregor, Ann. Mycol. 30 : 464. 1932.

Basidial fructification occurring as a delicate, closely appressed, often almost invisible film or weft of hyphae spreading over the apparently uninjured lower surface of the leaves of the host, usually observed as occurring at the margin or in advance of spreading necrotic lesions; sometimes developing sufficiently to form a delicate separable pellicle. The film or pellicle made up of intertwining, branching, thin-walled, simple-septate hyphae  $3.5\text{--}5.5\ \mu$  in diameter, branching often at right angles. Basidia short cylindric, broadly clavate, obovate, or irregular,  $10\text{--}18 \times 8\text{--}12\ \mu$ , often formed directly from hyphal cells when they may have a truncate base drawn out at either side or to one side if formed terminally. "Epibasidia", usually four, occasionally three, developing at first as subglobose projections, becoming cylindric and finally terete, arcuate, or rather widely divergent,  $10\text{--}16\ \mu$  long. Basidiospores asymmetrically ellipsoid, broadest below the middle, flattened and appearing straight on one side, with prominent lateral apiculus, varying somewhat in size on different hosts,  $9\text{--}13 \times 4.5\text{--}7\ \mu$ , germinating by repetition; walls thin, smooth, nonamyloid (Fig. 1).

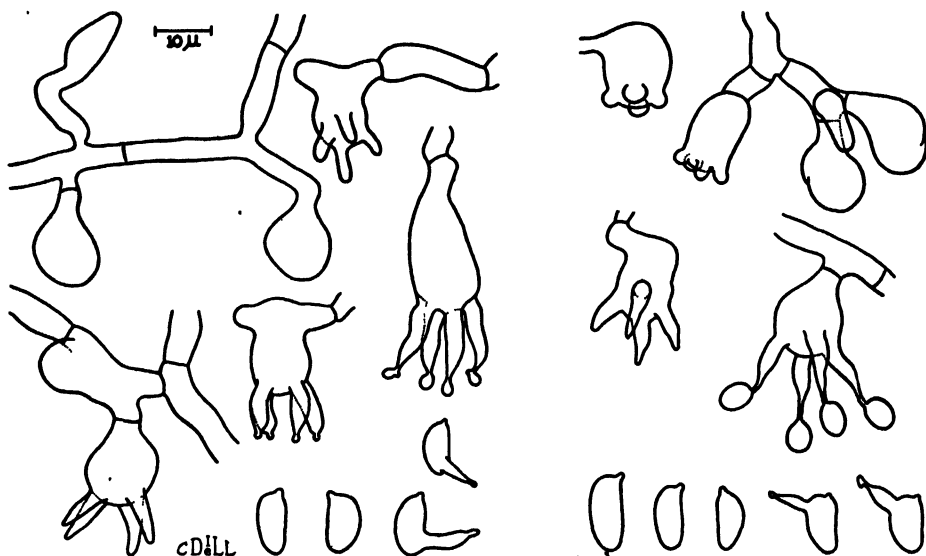


FIG. 1. *Ceratobasidium anceps*, hyphae, basidia, and spores. The group at left from *Aralia nudicaulis*, at the right from *Pteridium aquilinum* var. *latiusculum*.

. Following the advancing basidial fructification and often beginning to appear before basidial production has ceased, the hyphae become aggregated at close intervals into what may be termed appressorial pads or infection cushions (Gregor). As these develop the hyphae intertwine closely with septa at frequent intervals. At maturity a compact palisadelike layer of parallel hyphal tips develops on the side next the host epidermis with looser intertwined threads on the free side. Necrotic tissue develops as the cushions

mature and the latter persist on the dead areas as brownish specks and furnish a positive means of identification after the basidial fructification has disappeared.

Finally white fluffy masses of mycelium are developed on the surface of the dead areas at irregular intervals and these are gradually transformed into the characteristic brown sclerotia which, when fully developed, are deciduous.

Care must be taken not to confuse *C. anceps* with another related form which is not uncommon. One occasionally finds a *Corticium* in fruiting condition on the bases of stems of living ferns or of angiosperms. Sometimes this fungus may form a very delicate whitish film of mycelium and basidia not only on the stems or petioles but extending over considerable areas of the under surface of the living leaves. There is however in such cases no evidence of parasitism. The fungus is apparently growing superficially over the host, presumably spreading as a saprophyte from surrounding humus. No mycelial pads or sclerotia such as occur in *C. anceps* are formed and the host appears not to be affected. We have collections of this form from Bear Island, Lake Timagami, on the bases of stems of *Pteridium aquilinum* var. *latiusculum*, *Dryopteris Phegopteris*, *Onoclea sensibilis*, *Aralia nudicaulis*, *Diervilla Lonicera*, *Aster macrophyllus*, seedling *Fraxinus nigra*, and on stems and leaves of *Viola* sp., *Rubus pubescens*, and on an unidentified grass. A collection of the same form was also made at Inlet, New York, in 1934 on petioles and leaves of *Rubus pubescens* and on rachis and fronds of *Onoclea sensibilis*. While showing some variation in spore size and diameter of mycelium these collections are all referred, for the present, to *Ceratobasidium cornigerum* (Bourd.) Rogers (= *Corticium cornigerum* Bourd.) which, as at present interpreted, is also commonly found as a saprophyte on dead bark and wood of various trees. This species does not form sclerotia or appressorial pads in culture. It is quite different from *C. Solani* though fruiting in a similar relation to the host and has perhaps sometimes been confused with that species. Mrs. Gregor (7, p. 403) refers briefly to another *Corticium* which she found at the base of bracken fronds in connection with her studies of *C. anceps*, which appeared to be quite harmless. It is possible that this may also have been *C. cornigerum*, though no description was furnished, and the species was not identified.

### Hosts and Distribution

Our own collections in Ontario have been made entirely in the Timagami Forest Reserve. Most of these collections have been made in a small area about 50 yd. long along a trail on Bear Island. The fungus was also observed in abundance in the vicinity of Duchesnay, Que., at the time of the Mycological Society Foray held in late August 1938. It was particularly abundant there on *Dennstaedtia punctilobula* but present, for the most part, in the late sclerotial phase. Collections were made on seven hosts and had the observations been made a month earlier no doubt many other hosts could have been found. More recently other collections from Quebec have been made by Dr. S. M. Pady in the vicinity of Lennoxville and near Nemaygo. A number



of collections were made by the late Prof. H. H. Whetzel and students in Maine, New Hampshire, and New York. These collections, together with those made by Dr. Davis in Wisconsin discussed previously, form the basis of the host list in Table I.

TABLE I  
HOSTS AND DISTRIBUTION\*

Pteridophytes		Dicotyledons—concluded	
<i>Adiantum pedatum</i> L.	W, Q	<i>Hydrophyllum canadense</i> L.	NY
<i>Athyrium Filix-femina</i> (L.) Roth var.		<i>Hydrophyllum virginianum</i> L.	W, NY
<i>Michauxii</i> (Spreng.) Farwell	O	<i>Lactuca biennis</i> (Moench) Fern.	O
<i>Cystopteris bulbifera</i> (L.) Bernh.	Q	<i>Linnaea borealis</i> L. var. <i>americana</i> (Forbes) Rehd.	O
<i>Dennstaedtia punctilobula</i> (Michx.) Moore	Q, M	<i>Lonicera canadensis</i> Marsh. ?	O
<i>Dryopteris disjuncta</i> (Rupr.) Morton	O	<i>Lycopodium americanum</i> Muhl.	O
<i>Dryopteris Phegopteris</i> (L.) C. Chr.	O	<i>Lysimachia ciliata</i> L.	W
<i>Dryopteris spinulosa</i> (O. F. Muell.) Watt.	O, Q	<i>Mitella diphylla</i> L.	W
<i>Dryopteris Thelypteris</i> (L.) Gray var. <i>pubescens</i> (Lawson) Prince	NH	<i>Mitella nuda</i> L.	O
<i>Onoclea sensibilis</i> L.	O, W, NY	<i>Petasites palmatus</i> (Ait.) Gray	O
<i>Pteris pennsylvanica</i> (Willd.) Fern.	O, W	<i>Plantago major</i> L.	O
<i>Pteridium aquilinum</i> (L.) Kuhn var. <i>latiusculum</i> (Desv.) Underw.	O, Q, W	<i>Prenanthes</i> sp.	Q
† Dicotyledons		<i>Prunella vulgaris</i> L.	O
<i>Acer spicatum</i> Lam.	O	<i>Pyrola elliptica</i> Nutt. ?	O
<i>Acer</i> sp. (seedling)	Q	<i>Ranunculus septentrionalis</i> Poir.	W
<i>Achillea Millefolium</i> L.	O	<i>Ranunculus acris</i> L. ?	O
<i>Apocynum androsaemifolium</i> L.	O	<i>Ribes glandulosum</i> Grauer	O
<i>Aralia nudicaulis</i> L.	O, Q, W, NY	<i>Rubus allegheniensis</i> Porter	W
<i>Aster macrophyllus</i> L.	O	<i>Rubus strigosus</i> Michx.	O, Q
<i>Aster</i> sp.	W	<i>Rubus pubescens</i> Raf.	O
<i>Bidens frondosa</i> L.	W	<i>Silphium terebinthinaceum</i> Jacq.	W
<i>Chrysanthemum Leucanti' emum</i> L.	O	<i>Solidago canadensis</i> L.	W
<i>Cornus canadensis</i> L.	O, Q, NH	<i>Solidago gigantea</i> Ait. var. <i>leiophylla</i> Fern.	W
<i>Corylus cornuta</i> Marsh.	O	<i>Taraxacum officinale</i> Weber	O
<i>Dicentra Cucullaria</i> (L.) Bernh.	Q	<i>Tiarella cordifolia</i> L.	Q
<i>Diervilla Lonicera</i> Mill.	O, W	<i>Trientalis borealis</i> Raf.	O, W, Q
<i>Epilobium angustifolium</i> L.	O	<i>Viburnum alnifolium</i> Marsh	Q
<i>Epilobium glandulosum</i> Lehm. var. <i>adenocaulon</i> (Hausk.) Fern.	O	<i>Viola</i> sp.	O
<i>Fragaria vesca</i> L. var. <i>americana</i> Porter	O	Monocotyledons	
<i>Fragaria virginiana</i> Duch.	W	<i>Carex acutata</i> Boot. ?	O
<i>Galium triflorum</i> Michx.	O	<i>Clintonia borealis</i> (Ait.) Raf.	O, Q
<i>Hachelia virginiana</i> (L.) I. M. Johnston	O	<i>Habenaria viridis</i> (L.) R. Br. var. <i>bracteata</i> (Muhl.) Gray	O
<i>Hieracium scabrum</i> Michx. ?	Q	<i>Maianthemum canadense</i> Desf.	O, Q
		<i>Streptopus roseus</i> Michx.	O
		<i>Trillium cernuum</i> L.	O
		<i>Trillium</i> sp.	Q

\* O = Ontario, Q = Quebec, M = Maine, NH = New Hampshire, NY = New York, W = Wisconsin. All collections on which the above list is based are represented in the mycological herbarium of the University of Toronto, TRT.

### Symptomology

Mrs. Gregor (7, pp. 403-406) has given an excellent account of the development of the infected areas and of the symptoms of the disease as it occurs on bracken. Our own observations on ferns correspond very closely with her account. In general the ultimate effect of infection is quite similar on the different species. Older infected areas of the pinnae become brittle and

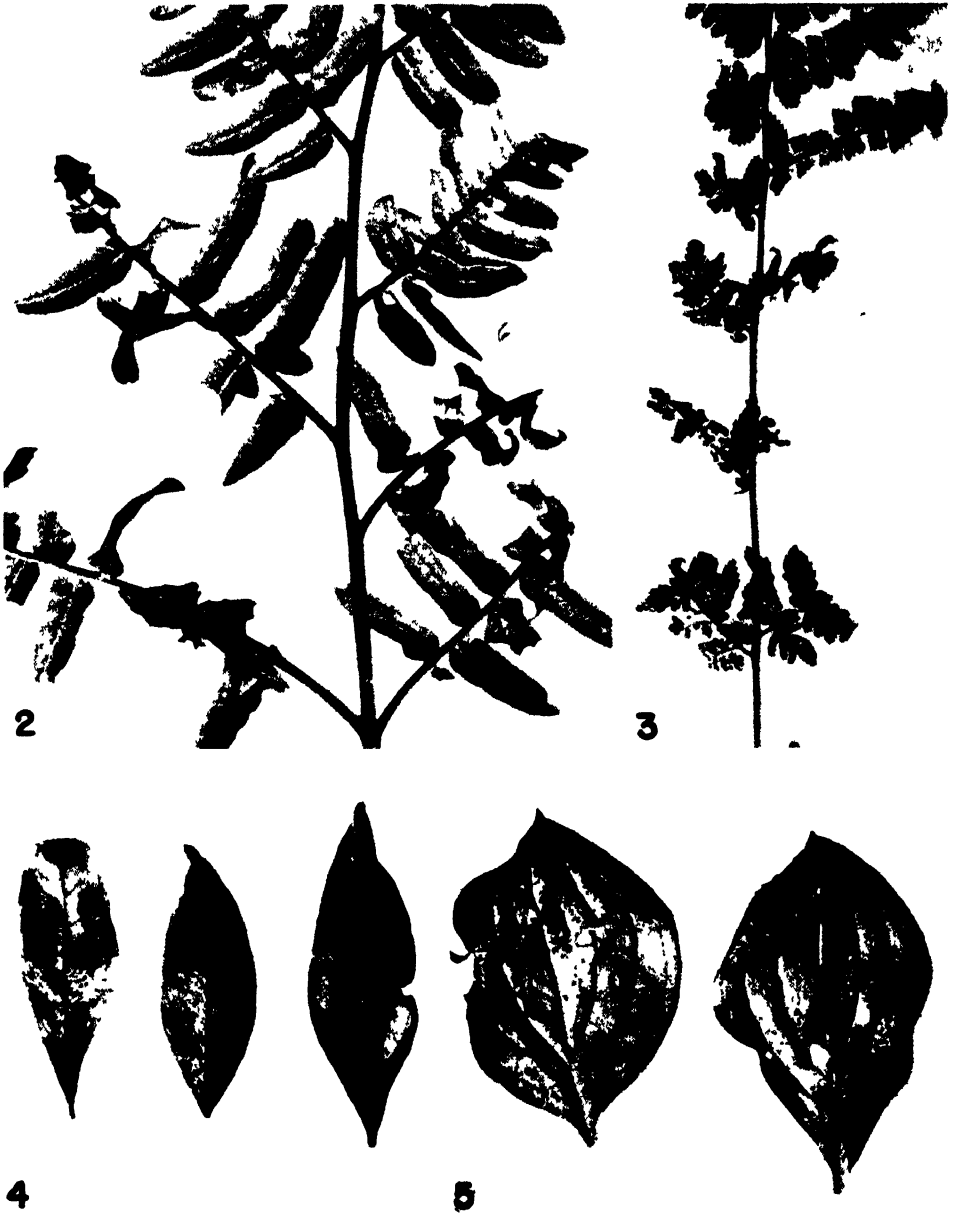


FIG. 2. Result of infection by *Ceratobasidium anceps* on *Pteridium aquilinum* var. *latiusculum*. Natural size.

FIG. 3. Same, on *Dryopteris spinulosa*

FIG. 4. Same, on *Trientalis borealis*, the two leaflets at left show the under surface, the one at right the upper surface. Note appressorial pads on lesions at left

FIG. 5. Same, on *Cornus canadensis*, lower surface showing lesions with appressorial pads.



FIGS. 6, 7, 8. Infection of *Ceratobasidium anceps* on *Clintonia borealis* proceeding from tip of leaf, downward. Note appressorial pads and sclerotia in Fig. 6. Young and mature appressorial pads are shown in Fig. 7. Fig. 8 is an enlargement  $\times 2$  of a portion of the leaf at right of Fig. 6 and shows young appressorial pads. The basoidal fructification is present below the pads on the uninjured leaf surface.

usually tend to drop away leaving a more or less naked rachis. This was particularly noted in the case of *Dennstaedtia punctilobula* in the vicinity of Dushesnay, Que. In one locality, in a moist low woods, infection by *C. anceps* had reduced the leafy portions of the fronds almost completely over a considerable area. Infected fronds of *Pteridium aquilinum* var. *latiusculum* and of *Dryopteris spinulosa* are shown in Figs. 2 and 3.

On the angiosperms the gross symptoms are very variable on the different species attacked. The necrotic areas vary in size, shape, and coloring, though some shade of brown is predominant. Illustrations (Figs. 4 to 10) of the effect of the fungus on several hosts will be found in Plates I to III and the following account deals primarily with those examples.

On *Clintonia borealis* (Figs. 6, 7, 8), infections are frequently at the tip of the leaf, proceeding downwards. In advance of necrosis, an inconspicuous basidial phase may be perceived with the aid of a lens as a delicate pelliculose film on the lower surface of the leaf. Appressorial pads are first visible beyond the necrotic margin as minute white thickenings of the surface mycelium (Fig. 8), which at maturity are dark brown and thickly scattered over the injured portion of the leaf (Fig. 7). Sclerotia, beginning as fluffy masses of hyphae, are relatively abundant. The infected tissue becomes discolored, finally brown, a blackish green, or almost colorless and may become brittle and drop away.

On *Aralia nudicaulis* (Figs. 9, 10), lesions are diffuse, finally involving all green tissue of an infected leaf. The infections are irregularly spreading, on the lower surface conspicuously punctate from the appressorial pads. The advancing margin of necrosis is sharply delimited, and often a dark sepia brown. Behind the margin, the killed tissue may be variously colored, fading rapidly to a buff or olive green, ultimately becoming straw colored or almost colorless, the upper surface more intensely colored than the lower. The gross appearance of heavily infected leaves varies considerably. Many are marked by large spreading islands of necrotic tissue, each sharply delimited and separated one from the other by bands of green tissue; many others have one necrotic area merging with another, the whole a mosaic of discolored, mottled, dead tissue.

On *Trientalis americana* (Fig. 4), infected areas are frequently reddish brown and sufficiently thin and transparent that the appressorial pads are as conspicuous from the upper surface as from the lower. Young infections are spotty, orbicular, but spreading and eventually involving the entire leaf. The apparently uninjured tissue of infected leaves may turn reddish. This same reaction has been observed on collections of *Cornus canadensis* (Fig. 5) made late in August and September. This may be a seasonal variation co-related with the maturation of the host, for July collections do not show the same discoloration. Lesions on *Cornus* are sharply differentiated, brown or buff in the center, becoming white, and surrounded on the upper surface by a narrow purplish rim. Leaves of late collections may be entirely reddish purple in advance of necrosis.

On *Maianthemum canadense* and *Apocynum androsaemifolium* much of the green tissue becomes chlorotic in advance of spreading necrosis; in *Acer spicatum*, *Clintonia borealis*, and *Diervilla Lonicera* chlorosis is less evident. This variation in symptomology has been described on the basis of dried herbarium specimens collected over several seasons. Controlled infection experiments would provide valuable data on the progress of the disease and the reaction to the disease by selected hosts. However, the presence of appressorial pads on necrotic lesions of a host is apparently indisputable evidence of the parasitic phase of this fungus.

### Cultures

Pure cultures are readily obtained from germinating basidiospores or by direct transfer of the appressorial pads to poured agar plates. In the latter case subtransfers from the margin of the advancing colony will give pure cultures, though often the original transfer appears to be pure. We have not made any extensive study of cultures but have verified most of the observations recorded by Mrs. Gregor (7). Cultures obtained from the fungus on different hosts do not vary appreciably so far as we have observed them. In Fig. 12 a photograph of a Petri dish is shown in which colonies originating from cultures obtained from six different hosts were grown side by side. The central colony originated from *Pteridium aquilinum* var. *latiusculum*. Starting at the top and reading clockwise the outer ring of colonies originated from *Clintonia borealis*, *Taraxacum officinale*, *Dryopteris spinulosa*, *Clintonia borealis*, *Cornus canadensis*, and *Streptopus roseus*. No appreciable difference in these colonies was noted at this stage of growth or later. In Fig. 11, two plates are shown in which the fungus has been allowed to grow until the whole plate is covered. The upper one originated from a culture obtained from *Streptopus roseus* and the lower one from *Pteridium aquilinum* var. *latiusculum*. The latter culture compares very favorably with Mrs. Gregor's Fig. 10 except that our culture shows more evident zonation. This difference may have been due to the conditions under which the plates were handled during the period of growth. More sclerotia were formed in the culture from the *Pteridium* as compared with the one from *Streptopus* but, as suggested by Mrs. Gregor, such a difference may have been due to the depth of the medium. Appressorial pads developed abundantly on the sides of the culture dishes or tubes as noted by Mrs. Gregor in her cultural studies.

No basidia have been observed to form in culture either by Mrs. Gregor or the writer. It seems probable that they would develop in polysporous cultures

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FIGS. 9 and 10. Infection of *Ceratobasidium anceps* on *Aralia nudicaulis* to illustrate the extensive lesions. Fig. 9 shows the upper surface, Fig. 10 the lower. Note mottled effect on the upper surface.

FIG. 11. Cultures of *Ceratobasidium anceps*. The upper plate from *Streptopus roseus*, the lower one from *Pteridium aquilinum* var. *latiusculum*.

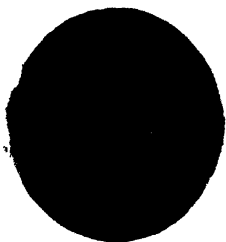
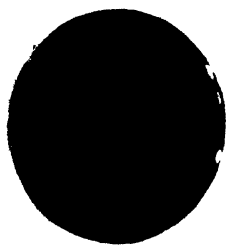
FIG. 12. Plate with colonies of *Ceratobasidium anceps* obtained from six different hosts. See text.



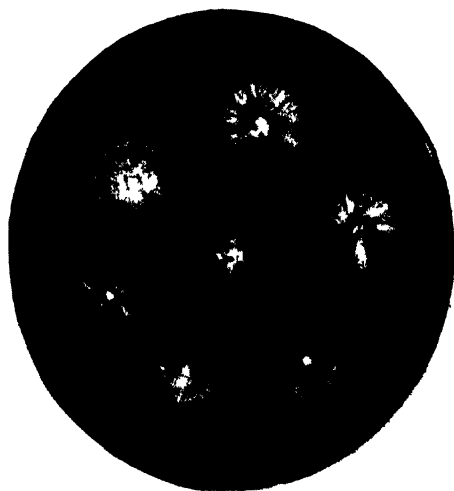
9



10



11



12



or in cultures from appressorial pads or sclerotia if the proper conditions were provided. Whether they would develop in cultures obtained from single basidiospores might depend on whether or not the fungus is homothallic or heterothallic, a problem which has not yet been investigated.

### Discussion

The primary purpose of the present contribution has been to record the occurrence, host range, and distribution of *Corticium anceps* in North America. Only preliminary observations have been made with reference to other intriguing problems which have become obvious from our admittedly incomplete study of this very interesting parasite. It seems desirable, however, to review these problems briefly and present what is known about them as a basis for future study.

The fact that the fungus is recorded in Europe and Great Britain only on ferns while our observations indicate a wide host range, immediately raises the question of the possibility of the existence of host specialized forms or races. Is the form studied by Mrs. Gregor more restricted in host range than the one occurring at Bear Island, Timagami, where our own observations have been made? Is there more than one strain present in the latter region? Will isolates of the fungus obtained in other North American localities prove comparable in host relations to those to be obtained in the Bear Island area? The fact that the fungus has not been found on other hosts than ferns in Europe and Great Britain is suggestive that the race occurring there is more restricted in host range than the one or more races occurring in N. America. On the other hand it is possible that infections on angiosperms have been overlooked. Mrs. Gregor (7), as noted above, in controlled inoculation experiments, was able to transfer the fungus from bracken to eight other fern hosts. Unfortunately she attempted inoculation on only two species of angiosperms, both exotic species—*Solanum tuberosum* and *Lycopersicon esculentum*—and did not attempt inoculation on any native plants. The two solanaceous hosts were selected because of the assumption that *C. anceps* was closely related to *Corticium Solani* (Prill. & Del.) Bourd. & Galz. and presumably also because Bourdot and Galzin (1, p. 242) had confused *Tulasnella anceps* with *C. vagum* Berk. & Curt. with which species Burt (2, 3) had included *C. Solani*. These inoculations gave negative results.

As noted in the introduction, our first observations of the disease were made on angiosperms. A summary of our collections to date shows that in a relatively small area on Bear Island in Lake Timagami the fungus has been found on a total of 41 hosts, including 7 species of pteridophytes, 6 monocotyledons, and 28 dicotyledons (see Table 1, p. 246). No entirely satisfactory controlled inoculation experiments have yet been conducted. However, in May 1947 a preliminary series of inoculations was made by Miss E. Ruth Dearden in the greenhouse at the Department of Botany, Toronto, using cultures obtained at Bear Island, Timagami, in the summer of 1946. From a culture obtained from the fungus on *Pteridium aquilinum* var. *latiusculum*,



infection with incipient necrotic lesions and the development of appressorial pads was obtained on cultivated bean (*Phaseolus vulgaris*), tomato (*Lycopersicon esculentum*), and on an exotic species of *Pteris*. Spreading of the fungus from the inoculum with the development of appressorial pads was obtained on corn (*Zea Mays*) but no necrotic lesion developed. From a culture isolated from *Aralia nudicaulis*, incipient necrotic lesions with the development of appressorial pads was obtained on the *Pteris*, bean, tomato, and corn. Basidia were not developed in any of these experiments. The conditions necessary for infection and the normal development of the basidial fructification and of typical lesions are evidently rather exacting and were not duplicated with entire success. Field inoculations, not controlled or protected, made by applying diseased foliage to apparently healthy leaves of other hosts have been made as follows: from *Aralia nudicaulis* to *Pteridium aquilinum* var. *latiusculum*; from the latter to *Clintonia borealis* and to *Rubus strigosus*. These preliminary inoculation experiments are not interpreted as conclusive but seem to indicate that the fungus occurring at Bear Island may be transferable from ferns to angiosperms and vice versa.

The writer has inclined to the view, based primarily on field observations over a period of 14 years, that the fungus in the Bear Island area is primarily of one strain. The chief basis for this view, inconclusively supported by the preliminary inoculation experiments mentioned above, has been the occurrence of the fungus on a wide host range in a relatively small area, together with the fact that infections of the fungus have frequently been observed in the field passing from one host to another where the foliage is in contact. Following is a list of such cases that have been observed and of which specimens have been preserved. The infection appeared to be progressing from the one host to the other as indicated:

- from *Dryopteris spinulosa* to *Lactuca spicata*
- from *Dryopteris disjuncta* to *Ribes prostratum*
- from *Pteris pennsylvanica* to *Aralia nudicaulis*
- from *Aralia nudicaulis* to *Corylus rostrata*
- from *Aralia nudicaulis* to *Petasites palmatus*
- from *Cornus canadensis* to *Corylus rostrata*.

Controlled inoculations on a wide variety of native plants using cultures from various hosts and localities in North America should ultimately be made in comparison with cultures obtained from bracken in Scotland. Until this is done the problem of the existence of host specialized races cannot be finally answered.

The sequence of events as observed in connection with the development of the mature lesions is suggestive of an interesting type of parasitism. As noted above, the basidial fructification appears to develop for the most part on the apparently uninjured surfaces of the leaves. It is easily stripped off as a thin film and no evidence is available that it forms any direct contact with the epidermal cells of the host. In the case of older infections this film

is apparently dependent for its development primarily on food available from the hyphae in the necrotic lesions though there is a possibility that substances associated with the outer layers of the cuticle may furnish some sustenance. Initial local infections previous to the development of necrotic lesions present a special problem which requires further investigation. In August, and later basidia are seldom found but an advancing film of sterile hyphae may be present in actively spreading infections.

From a study of microtome sections and of whole mounts cleared in lactophenol and stained with cotton blue, no evidence was obtained of the presence of mycelium within the leaf beneath the advancing basidia bearing hyphae, until the appressorial pads begin to develop. The presence of intercellular hyphae in the tissues can be observed soon after the young appressorial pads are recognizable. On present evidence these structures appear to be directly related to the colonization of the tissues by the fungus. As has been stated by Mrs. Gregor (7), direct penetration by hyphae arising from the underside of the appressorial pads can readily be demonstrated in microtome sections. Further study is needed to determine whether or not infection may also occur through stomates.

There are several aspects of the general biology of the fungus which have not been fully determined. The sclerotia which ultimately form in connection with the host lesions and which develop abundantly in culture quite certainly perform an important function in the life cycle. These sclerotia are deciduous when fully formed and drop to the ground. Cultures of the fungus may be readily obtained from sclerotia developed in culture or on the host. Mrs. Gregor (7) has shown that sclerotia are viable after overwintering on the bracken and hence are capable of carrying the fungus over the winter.

The further history to the time of initial infection is unknown. Do the sclerotia develop to a saprophytic growth with production of basidia from which air-borne spores are disseminated and cause the initial infections directly on the pinnae or leaves of the host or does infection first occur directly from sterile mycelium as the fern fronds or the early growth of perennial angiosperms push through the ground in early spring? There would seem to be little doubt that the basidiospores are capable of spreading infection from plant to plant and perhaps from host to host during the early summer, but this problem needs further study.

Is the sole function of the appressorial pads that which is associated with the colonization of the tissues by the fungus or may they also function as microsclerotia and serve as a second means of carrying the fungus over from season to season?

The nuclear history of the fungus should ultimately be investigated and should be studied from cultures obtained from monospores as well as from polyspores or from the appressorial pads or sclerotia. The hyphae in the host tissue appear to be dikaryotic so far as observed.

Since clamps are not formed at the septa the usual method of determining whether the fungus is homo- or heterothallic is not available. The development of a method of obtaining consistent production of basidia in culture would be of great assistance in connection with the elucidation of this problem. If the species proves to be homothallic, where does the dikaryon arise?

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## THE INTERMEDIATE METABOLISM OF *PSEUDOMONAS AERUGINOSA*

### III. THE APPLICATION OF PAPER CHROMATOGRAPHY TO THE IDENTIFICATION OF GLUCONIC AND 2-KETOGLUCONIC ACIDS, INTERMEDIATES IN GLUCOSE OXIDATION<sup>1</sup>

BY FLORA C. NORRIS<sup>2</sup> AND JACK J. R. CAMPBELL<sup>3</sup>

#### Abstract

The technique of paper chromatography has been adapted to the identification of gluconic, 2-ketogluconic, and  $\alpha$ -ketoglutaric acids. Combinations of methyl and ethyl alcohol were found to be the most suitable solvents and ammoniacal silver nitrate was found to give the most satisfactory reaction. When grown under normal physiological conditions where glucose was metabolized to carbon dioxide and water, *Pseudomonas aeruginosa* 9027 was shown to have oxidized glucose by way of gluconic and 2-ketogluconic acids. Since a strong system for oxidizing both gluconic and 2-ketogluconic acids was demonstrated, the presence of these acids over at least an eight hour period of growth is taken as evidence of their importance as intermediates in the oxidation of glucose by this organism.

The meager information available on the intermediate products formed during the degradation of glucose by oxidative organisms such as *Pseudomonas aeruginosa* has been obtained under rigorous conditions usually on a commercial scale and the data available from these studies are thus not necessarily valid under normal physiological conditions. Pervozvanskii (9) has reported high yields of gluconic and ketogluconic acids during glucose oxidation by fluorescing bacteria when conditions of intense aeration and heavy concentrations of substrate were employed. In similar investigations Lockwood, Tabenkin, and Ward (5) have compared a number of cultures of *Phytomonas* and *Pseudomonas* with respect to their abilities to produce gluconic and 2-ketogluconic acids. Yields of 58 to 96% gluconic acid and greater than 70% 2-ketogluconic acid were obtained under commercial conditions although only traces of these compounds were produced under conditions in which aeration was minimal. *Pseudomonas aeruginosa* 9027, the organism used in the present work, has also been shown to produce appreciable quantities of 2-ketogluconic acid from glucose under conditions of intense aeration (6). Lockwood and Stodola (4) demonstrated another possible step in the dissimilation of glucose when they isolated  $\alpha$ -ketoglutaric acid during submerged fermentations of glucose, gluconate, or 2-ketogluconate by *Pseudomonas fluorescens*. Under the conditions of these experiments, however, the acids appear as end products and although they indicate a mechanism available to the cells they are not necessarily intermediate products in the normal oxidation of glucose to carbon dioxide and water.

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A study of the intermediate metabolism of an oxidative organism such as *P. aeruginosa* presents many difficulties. The organism oxidizes its substrate rapidly, and in general completely, to carbon dioxide and water, and, because of this strong oxidative power, under normal physiological conditions few of the intermediate compounds accumulate in sufficient quantity to be isolated and identified in the usual manner. In dealing with an organism about which so little is known, it did not seem justifiable to resort to the use of specific enzyme inhibitors or aeration as means of bringing about the accumulation of incompletely oxidized products. The technique of simultaneous adaptation (10), which appeared to be a useful tool for studying this organism, has been found to have serious limitations (1). Consequently it was necessary to employ a procedure that would separate and identify closely related compounds when present in only trace amounts. The technique of paper chromatography (2) suggested itself in this connection.

The purpose of the present study was to apply the technique of paper chromatography to the identification of the initial intermediate compounds formed when glucose is dissimilated by *P. aeruginosa* under normal physiological conditions.

### Methods

The culture of *P. aeruginosa* ATC 9027 employed was a typical strongly pigmenting strain. Stocks were maintained on liver gelatin agar and refrigerated after growth had been initiated at 30° C. Medium for growth of the organism was prepared as follows: ammonium dihydrogen phosphate 0.3%, magnesium sulphate septahydrate 0.1%, and iron 0.5 p.p.m. (as ferrous sulphate) were adjusted to pH 7.2, dispensed in 100 ml. quantities in Roux flasks, and sterilized by autoclaving at 15 lb. pressure for 15 min. Before flasks were inoculated, glucose that had been sterilized through sintered glass as a 50% solution was added aseptically to a final concentration of 0.5% and dipotassium hydrogen phosphate that had been sterilized by autoclaving as a 10% solution was added to a final concentration of 0.3%. Inoculum consisted of 1.0% of a 24 hr. culture grown in glucose ammonium phosphate medium. After subculture from the refrigerated stock the culture was always transferred at least twice at 24-hr. intervals in glucose ammonium phosphate medium before being used as inoculum.

For the preparation of concentrated supernatants, the organism was grown in 200 ml. of glucose ammonium phosphate medium at 30° C. for either 16 or 24 hr. before the cells were removed by centrifugation. The clear supernatant was adjusted to pH 7.2 with 4 *N* sodium hydroxide and then heated in a boiling water bath for five minutes to aid in suppressing further enzyme action. The supernatant was cooled and immediately vacuum distilled under nitrogen at approximately 30° C. until the final volume was 20 ml. Since the organism has been shown to be a strict aerobe (6) this method of concentrating under nitrogen was employed in order to prevent further dissimilation of glucose during the bubbling necessarily accompanying vacuum distillation.

A control flask containing 200 ml. of uninoculated medium was treated in a similar manner.

Paper chromatography was carried out both by the more conventional descending method (2) and by the ascending method (11). The latter method was used in exploratory work while the former was used in the subsequent more exact determinations. In the ascending chromatograms the compounds being tested were introduced as drops from a Pasteur pipette; these drops were placed at intervals  $1\frac{1}{2}$  in. from the bottom of a sheet of Whatman No. 1 filter paper which had been cut to measure 13 in.  $\times$  22 in. The sheet was pinned in the form of a cylinder and set in a large Petri dish containing 25 ml. of solvent. The cylinder and dish were placed in a clean wastebasket, a wooden cover was put in place and the sheet was irrigated at room temperature for 15 to 18 hr. The sheet was then dried and sprayed with 0.1 *N* silver nitrate in 5 *N* ammonium hydroxide. The sprayed sheet was allowed to dry in the absence of direct light. In the descending chromatograms, drops of the two supernatants, the concentrated medium and known compounds (2 mgm. per ml.) were introduced near the top of a sheet of Whatman No. 1 (18 in  $\times$  22 in.) filter paper which was hung vertically from a trough. The trough and filter paper sheets were then suspended inside a closed vessel whose atmosphere was kept saturated with respect to the solvent. The trough was filled with the solvent and sheets were irrigated at room temperature to within 2 in. of the bottom. The sheets were dried and sprayed with silver nitrate solution. The sprayed sheets were then dried.

The ethyl and methyl alcohols used as solvents were purified prior to use by refluxing with zinc dust in an alkaline solution for 60 min. before redistillation. Phenylhydrazones were prepared by allowing solutions to stand at room temperature overnight in contact with an equal volume of 10% phenylhydrazine hydrochloride. Ether extracts were prepared by adjusting solutions to pH 5.5 and extracting with ether for six hours in a continuous extractor. The methods used in Warburg work are those described in previous papers (1, 7).

### Experimental

Although the only information available on the use of paper chromatography for the study of intermediate carbohydrate metabolism was a preliminary report by Forsyth and Webley (3) on the hydrolysis of sucrose to the constituent hexoses by *Leuconostoc mesenteroides*, exploratory studies with *Pseudomonas aeruginosa* indicated that the technique would be applicable to the compounds and the conditions used in the present study.

The following compounds were found to give a definite spot with the silver nitrate reagent: glucose, glucose-1-phosphate, glucose-6-phosphate, hexose diphosphate, gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid,  $\alpha$ -ketoglutaric acid, pyruvic acid, lactic acid, malic acid, citric acid, glycerol, glycerol phosphate, and fructose.  $R_F$  values of these compounds are recorded in Table I. No reduction was given by succinic acid, fumaric acid, maleic acid,

TABLE I\*

$R_F$  VALUES OF KNOWN COMPOUNDS (2 MGM. PER ML.) IN 45% METHYL ALCOHOL, 45% ETHYL ALCOHOL, AND 10% WATER

Compound	$R_F$	Remarks
Glucose	0.59	
Glucose-1-phosphate	0.72	
Glucose-6-phosphate	0.36	
Hexose diphosphate	0.03	
Gluconic acid	0.425	
2-Ketogluconic acid	0.45	
5-Ketogluconic acid	0.45	This compound always gave two spots. It was not considered a possible intermediate since the organism could not grow with this compound as carbon source
	0.03	
$\alpha$ -Ketoglutaric acid	0.46	
Pyruvic acid	0.70	
Lactic acid	0.65	Yellow spot
Glycerol	0.76	
Malic acid	0.60	Orange-brown spot
Fructose	0.63	Grayish-brown spot
Citric acid	0.28	Orange spot
Glycerol phosphate	0.51	Faint orange spot

\* We are indebted to Dr. L. B. Lockwood of the Northern Regional Research Laboratory for samples of 2-ketogluconic and 5-ketogluconic acids; to Dr. C. E. Georgi of the University of Nebraska for a sample of 5-ketogluconic acid; to Dr. D. E. Green of the University of Wisconsin for a sample of  $\alpha$ -ketoglutaric acid and to Dr. I. C. Gunsalus of the University of Indiana for samples of glucose-1-phosphate, glucose-6-phosphate, and hexose diphosphate.

malonic acid, acetic acid, formic acid, formaldehyde, or acetone dicarboxylate. The spots obtained with silver nitrate were not always due to the presence of metallic silver; for instance, lactic acid gave a yellowish spot while citrate gave an orange spot. The gluconic acid spot was initially a yellowish shade turning dark brown with age. Although the silver nitrate reaction was not specific, many of these compounds could, however, be identified by color and  $R_F$  values.

Analysis of the culture supernatants always revealed at least two spots. One corresponded to glucose and the second with a smaller  $R_F$  value to some glucose degradation product. From a comparison of the  $R_F$  values obtained for known compounds, it was found that the value for this lower spot corresponded to that for gluconic, 2-ketogluconic, or  $\alpha$ -ketoglutaric acid. Since the movement of these three acids was very similar in all the 55 combinations of solvents used, some supplementary means of separation had to be found.

Advantage was taken of the fact that 2-ketogluconic and  $\alpha$ -ketoglutaric acids react with keto fixatives, whereas gluconic acid does not. The influence of sodium bisulphite, phenylhydrazine hydrochloride, hydroxylamine hydrochloride, semicarbazide, and 2 : 4-dinitrophenylhydrazine on the movement of these compounds was determined. Phenylhydrazine hydrochloride was

selected as the fixative to be used in further work, since the  $R_F$  values of the derivatives that were formed with it differed most markedly from those of the free acids while the  $R_F$  value of gluconic acid remained unchanged. An equal volume of a 10% solution of phenylhydrazine hydrochloride was allowed to react for 24 hr. at room temperature with the solution to be tested. These conditions permitted the complete conversion of any reasonable amount of 2-ketogluconate or  $\alpha$ -ketoglutarate to its corresponding hydrazone.

The  $R_F$  values obtained, both before and after phenylhydrazine treatment, for known compounds and for the concentrated supernatant of a 24 hr. culture are recorded in Table II.

TABLE II  
MOVEMENT OF INTERMEDIATE COMPOUNDS WITH 45% METHYL ALCOHOL,  
45% ETHYL ALCOHOL, AND 10% WATER

	Before phenylhydrazine treatment	After phenylhydrazine treatment
(1) Glucose	0.55	0.66
(2) Gluconate	0.43	0.43
(3) 2-Ketogluconate	0.47	No spot
(4) $\alpha$ -Ketoglutarate	0.46	No spot
(5) Conc. uninoculated medium	0.55	0.66
(6) Conc. 24-hr. culture supernatant	0.45	0.43
(7) 1 : 1 dilution of (6)	{ 0.44 0.48	{ 0.46 No spot

In Tables II and III, the  $R_F$  values of spots corresponding to glucose in the supernatant and also those due to free phenylhydrazine have been omitted in order to minimize confusion. It can be clearly seen from these results (Table II) that the supernatant contained gluconic acid since a reducing spot with an  $R_F$  value corresponding to this compound remained after phenylhydrazine treatment. However, in each case the size of the spot was considerably reduced by phenylhydrazine treatment, indicating that some compound that would react with phenylhydrazine had been removed. On dilution of the concentrated supernatant with an equal volume of water, the larger single spot, which had an  $R_F$  of 0.45, was seen to actually be composed of two smaller spots, one with an  $R_F$  of 0.44 and the other with an  $R_F$  of 0.48. The spot with an  $R_F$  of 0.44 was gluconic acid since it remained undiminished after phenylhydrazine treatment, while the spot with an  $R_F$  of 0.48 could be either 2-ketogluconic or  $\alpha$ -ketoglutaric acid or a mixture of these two compounds since it was eliminated by phenylhydrazine treatment.

In order to confirm the findings recorded in Table II, a second solvent containing 90% redistilled ethyl alcohol and 10% water was used (Table III).



TABLE III

MOVEMENT OF INTERMEDIATE COMPOUNDS WITH 90% ETHYL ALCOHOL AND 10% WATER

	$R_F$ values	
	Before phenylhydrazine treatment	After phenylhydrazine treatment
(1) Glucose	0.56	0.56
(2) Gluconate	0.33	0.33
(3) 2-Ketogluconate	0.33	No spot
(4) $\alpha$ -Ketoglutarate	0.32	No spot
(5) Conc. uninoculated medium	0.56	0.54
(6) Conc. 24-hr. culture supernatant	0.36	0.35
(7) Conc. 16-hr. culture supernatant	0.41	0.36

In this experiment, the concentrated supernatant from a 16 hr. culture was analyzed with a view to demonstrating the presence of the same intermediate compounds in young cultures. The lower reducing spot observed previously was again detected and it remained after treatment with phenylhydrazine but was reduced in size. These findings indicate the presence of gluconic acid and a keto acid in the 16 hr. supernatant as well as in the 24 hr. supernatant.

Further separation of reducing compounds was attempted on the basis of the relative solubilities of  $\alpha$ -ketoglutaric acid, 2-ketogluconic, and gluconic acids in ethyl ether. Gluconic acid, 2-ketogluconic acid,  $\alpha$ -ketoglutaric acid, concentrated uninoculated medium, concentrated 24 hr. supernatant, and concentrated 16 hr. supernatant were ether extracted for six hours at pH 5.5. The ether soluble and ether insoluble fractions of all solutions as well as fractions of each solution that had not been extracted were analyzed using a solvent consisting of 95% ethyl alcohol and 5% ammonium hydroxide.

TABLE IV

 $R_F$  VALUES OF ETHER FRACTIONS OF CULTURE CONSTITUENTS

Compound	$R_F$	$R_F$ ether insol. residue	$R_F$ ether sol. fraction
Glucose	0.42		
Gluconate	0.15	0.15	No spot
2-Ketogluconate	0.143	0.142	No spot
$\alpha$ -Ketoglutarate	0.056	No spot	0.06
Medium	0.40	0.38	No spot
24-Hr. supernatant	0.39	0.40	No spot
	0.135	0.142	
16-Hr. supernatant	0.39	0.42	No spot
	0.135	0.152	

The ether fractions were treated with 10% phenylhydrazine hydrochloride with results similar to those recorded in Tables II and III. Again the reducing

spot representing the intermediate products was reduced but not completely removed by the phenylhydrazine treatment, indicating that gluconic acid and 2-ketogluconic or  $\alpha$ -ketoglutaric acids or both were formed by the organism. Since no product could be detected in the ether extract of the culture, it can be concluded that  $\alpha$ -ketoglutarate was not present in detectable quantities, and that 2-ketogluconic acid was the keto acid present.

Further confirmation of the presence of gluconic acid in the culture supernatants was recorded in every experiment. Shortly after the chromatogram was sprayed with silver nitrate, gluconic acid came up as a characteristic yellow spot which later turned brown and a spot with the same characteristics and with the same  $R_F$  value always appeared in the culture supernatant.

Curves showing the ability of *P. aeruginosa* to oxidize gluconic and 2-ketogluconic acids are presented in Fig. 1. These data combined with the chromatographic analysis of the 16 and 24 hr. cultures indicate that gluconic and 2-ketogluconic acids are being formed and oxidized continually and that they are not just the end products of a minor side reaction.

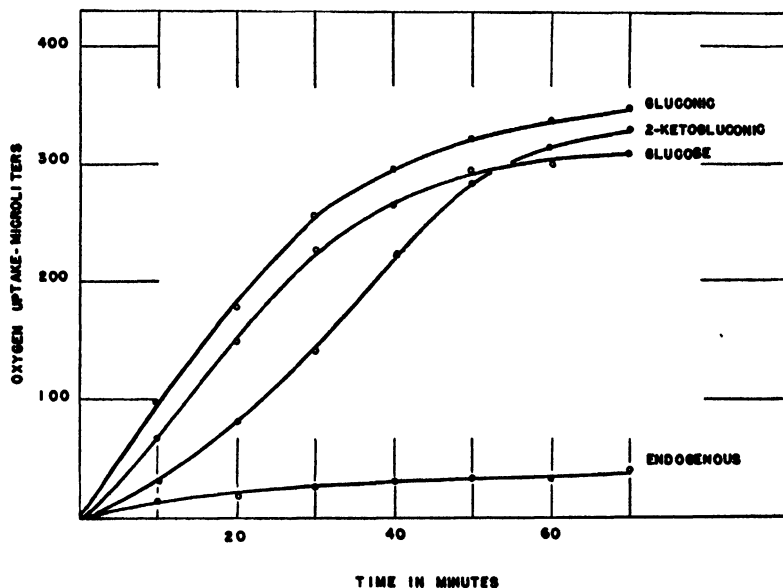


FIG. 1. Oxidation of glucose, gluconic, and 2-ketogluconic acids by 22-hr. cells harvested from glucose ammonium phosphate medium.

Warburg cups contained 0.5 ml. cell suspension, 1.5 ml. *M/15* phosphate buffer, pH 7.2, 0.2 ml. substrate. Final volume was 3.0 ml. Theoretical oxygen uptake for complete oxidation of any substrate was 403  $\mu$ l.

### Discussion

The use of paper chromatography for the detection of gluconic, 2-ketogluconic, or  $\alpha$ -ketoglutaric acids had not been previously reported and although a satisfactory procedure was eventually evolved, numerous difficulties were

encountered and a great deal of background information had to be accumulated. In searching for a solvent for the chromatographic analysis, one that would not interfere with the silver reduction reaction, would move the compounds being tested, and would be readily volatilized was essential. Saturated phenol, collidine, and butanol - acetic acid, the solvents commonly employed for the chromatographic analysis of reducing sugars (8), were found to be unsuitable as solvents since they did not move the possible degradation products of glucose through a sufficient distance to allow differentiation. It was found that methyl and ethyl alcohols at various concentrations, alone or in combination, served as excellent solvents for the compounds being tested. The alcohols were easily purified and they had the additional advantage of being volatile and thus readily removed.

The  $R_F$  values obtained were found to vary quite markedly with small differences in the water content of the solvent. It was therefore necessary to run known solutions with each individual chromatogram rather than use values obtained in previous determinations. Salts of acids or free acids were always found to give identical  $R_F$  values.

From the data presented it can be concluded that both gluconic and 2-ketogluconic acids were present in detectable amounts in a 16 hr. and 24 hr. culture of *P. aeruginosa* grown in glucose ammonium phosphate liquid medium. It was also found that in a 38 hr. culture all the glucose had disappeared and only the faintest trace of gluconic or 2-ketogluconic acid could be detected chromatographically. It has also been shown that *P. aeruginosa* has a strong mechanism for oxidizing both of these compounds. One must therefore conclude that these compounds are being formed at a continuous and vigorous rate and are being removed in the same manner. This can only mean that these compounds are part of the system through which most if not all of the glucose is oxidized.

The fact that cells harvested from a glucose medium require a period of adaptation before oxidizing 2-ketogluconic acid confirms the earlier evidence (1) that the technique of simultaneous adaptation is not applicable to studies such as these.

### Acknowledgment

The authors wish to express their appreciation to Dr. B. A. Eagles for his helpful suggestions and criticism of the manuscript.

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## X-RAY TOLERANCE OF LIVING CELLS AS MEASURED BY CYTOPLASMIC STREAMING<sup>1</sup>

BY CHARLES J. BISHOP,<sup>2</sup> VICTOR D. McLAUGHLIN,<sup>3</sup> AND DONALD F. TAPLEY<sup>3</sup>

### Abstract

An investigation was undertaken to determine the immediate effects of X radiation on living, nondividing cells using the rate of cytoplasmic streaming as a measure of cell vitality. The observations were carried out on stamen hairs and pollen tubes of *Tradescantia paludosa* (diploid) and *Tradescantia virginiana* (tetraploid). No significant variation in the rate of streaming of cytoplasm in either stamen hairs or pollen tubes was noted with low doses of X rays. In both diploid and tetraploid stamen hairs, 700,000 r. was required to stop all streaming. In the pollen tubes of diploid and tetraploid plants, all streaming was stopped by 250,000 r. and 200,000 r. respectively, a difference that was statistically significant. Greater sensitivity, as shown by resulting cell death, was found with an increased initial dosage of continuous irradiation.

### Introduction

The vitality of certain living plant cells may be determined by observing the rate of cytoplasmic streaming. In cells where this normally is visible, a decrease in the rate of streaming is considered indicative of lowered vitality, and a complete cessation is generally assumed to indicate death, accompanied by the ending of all normal cell functions.

The present investigation was undertaken to determine the immediate effects of X radiation on living, nondividing cells, using the rate of cytoplasmic streaming as an indication of cell vitality.

As early as 1898 small doses of X radiation were observed by Lopriore (4) to produce an acceleration in the movement in protoplasm. Similar results were noted by Williams (11) and Nadson and Rochlin (5), who, in addition, found that the period of acceleration was soon followed by signs of depression. Wanner (9) using root hairs of *Hydrocharis morsus ranae* and epidermal cells of *Allium Cepa* found that, after irradiation with 400 to 10,000 roentgens,\* there was an immediate decrease in viscosity of the protoplasm in epidermal cells of *Allium* and an increase in the rate of streaming in the root hairs of *Hydrocharis*. He found that the maximum speed increase in its absolute and relative magnitude showed no distinct dependence on the radiation dose, and that up to 6,000 r., the original speed recurred after a 10 hr. period of acceleration. Wanner also stated that, from 6000 to 12,000 r., permanent damage to the protoplasm and the ultimate death of the cell followed the period of acceleration. With doses exceeding 12,000 r., an immediate depression of

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\* A roentgen is the unit of measurement of radiation dosage established by international agreement for all scientific research.

the rate of streaming, followed by damage to the protoplasm, was noted. The dosage in Wanner's experiments was not increased to the point at which protoplasmic streaming stopped immediately, but rather an initial dose was administered and changes observed in viscosity (by centrifuging) or in the rate of streaming (by observation) for periods as great as 50 hr. The present investigations were carried to the point where sufficient X rays were administered to cause an immediate cessation of cytoplasmic streaming and thus, presumably, death of the cell.

### Materials and Methods

Cytoplasmic streaming was observed in the stamen hairs and pollen tubes of *Tradescantia paludosa*, a diploid species, and in *Tradescantia virginiana*, a tetraploid species. The rate of streaming, in microns per minute, was determined by observing the flow of cytoplasm, using a calibrated ocular micrometer. By this means a comparison was made between the radiation sensitivities of the diploid and tetraploid species.

The stamen hairs were cultured on an agar medium of 7.5% sucrose, 1% agar in distilled water. In both cases the medium was applied to a cover glass inverted over a Van Tieghem cell, which was used as a modified moist chamber (2). This technique made it possible to subject the material to long periods of irradiation without danger of the culture drying, and allowed observation of the cells with both low and high power objectives.

In each experiment cultures were first checked for rates of streaming and then subjected to a large uninterrupted dosage of radiation, followed by dosages of 25,000 r. each, until cytoplasmic streaming ceased. Observations were taken between each period of irradiation. For stamen hairs the initial dosage was 250,000 to 450,000 r., and for pollen tubes was 100,000 to 125,000 r.

The X radiation was from a standard Coolidge type X-ray tube having an inherent filtration of 1 mm. of aluminum and operated at 140 kv. and 5 ma. with a beam intensity of 1200 r.p.m. Calibrations were made with a Victoreen dosimeter, duplicating as nearly as possible the conditions under which the material was irradiated. In doing this, the ionization chamber of the dosimeter was placed above a microscope slide, surrounded on three sides by a glass arc, and covered by a cover glass of the same thickness used in the experiments.

### Results

In stamen hairs, no significant variation in the rate of cytoplasmic streaming was observed with low doses of radiation. In pollen tubes, the normal variation within a single cell was too great to permit the observation of a change in speed as small as that to be expected with the low doses.

Large doses of X rays invariably caused a cessation of cytoplasmic streaming both in stamen hairs and in pollen tubes. The effect of the X rays was found in all cases to be permanent. With pollen tubes, this is less significant because streaming normally stops after approximately 15 hr. On the other hand,

stamen hairs in which the rate of streaming had been reduced to  $150\ \mu$  per minute by irradiation remained unchanged for 96 hr. In another experiment, cells where activity had been reduced to Brownian movement were observed to be in the same condition at the end of 24 hr.

The results of experiments conducted on stamen hairs are presented graphically in Fig. 1. As indicated by the curves, the streaming became progressively slower up to 650,000 r. Thereafter, there was either no streaming at all or

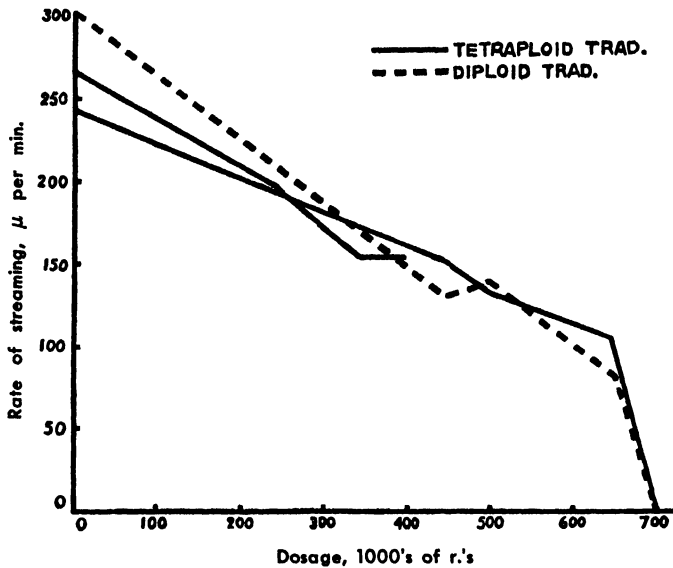


FIG. 1. The effect of X rays on cytoplasmic streaming in stamen hairs of diploid and tetraploid *Tradescantia*.

the streams were too short to permit accurate timing. Brownian movement was evident up to 750,000 r., but was not recorded as streaming. In the two experiments that were taken to completion, the initial dose as indicated by the graph was 450,000 r., whereas in the other experiment shown, the initial dose was 250,000 r. As shown by the graph, no significant difference was found between diploid and tetraploid stamen hairs.

In the case of pollen tubes, in which streaming normally stops after 15 to 20 hr. of growth, X rays caused a much earlier cessation of cytoplasmic streaming. There was no significant increase in the percentage bursting of the tubes, and their appearance was more normal than that of tubes dying a natural death, which usually have a granular appearance. There was no thickening of the walls of the pollen tubes as is frequently the case when death is normal.

The effect of X rays on cytoplasmic streaming in the pollen tubes of diploid and tetraploid plants is indicated in Fig. 2. This graph represents the average results of all the experiments conducted on pollen tubes. In none of the

experiments included in this graph were readings taken before 100,000 r. had been administered. From 100,000 to 250,000 r., readings were taken at intervals of 25,000 r., and each point on this graph has been determined by averaging approximately 45 readings. At each interval, a lapse of 10 min.

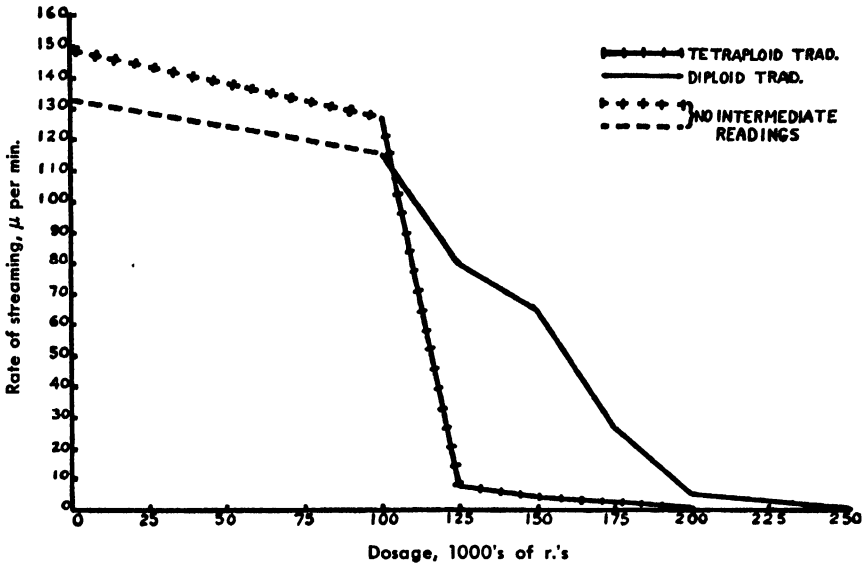


FIG. 2. The effect of X rays on cytoplasmic streaming in pollen tubes of diploid and tetraploid *Tradescantias*.

between irradiations was required to make accurate observations. A continuous irradiation of 125,000 r., administered to some of the pollen tubes of tetraploid *Tradescantias* frequently caused a complete cessation of streaming. This considerably lowered the average rate of streaming at this point for the whole series, and partially explains the sudden drop in the tetraploid curve between 100,000 and 125,000 r.

As is indicated by the graph, the maximum lethal dosage for the pollen tubes of tetraploid *Tradescantias* was 200,000 r., while for diploid plants it was 250,000 r. The average end point for tetraploid plants was  $154,625 \pm 10,500$  r., and for the diploids  $208,500 \pm 15,700$  r. A statistical analysis of the data shows that this difference is highly significant, indicating that in our experiments the diploid cells were more resistant than the tetraploid ones.

Fig. 3 is a graphical representation of one of the experiments included in the graph of Fig. 2. At each point where a reading is noted, there was a lapse of 10 min. between irradiations. In this experiment, all four cultures were made with the same medium and pollen from a single flower of each species was used. The graph indicates the difference between the pollen tubes of diploid and tetraploid *Tradescantias* and also shows the variation produced by changing the initial dose. Tubes from both the diploid and tetraploid



species given an initial dose of 100,000 r. required a greater amount of irradiation to stop the streaming than did those given an initial dose of 125,000 r. Thus it is shown that fractionation reduces the effectiveness of a given dose.

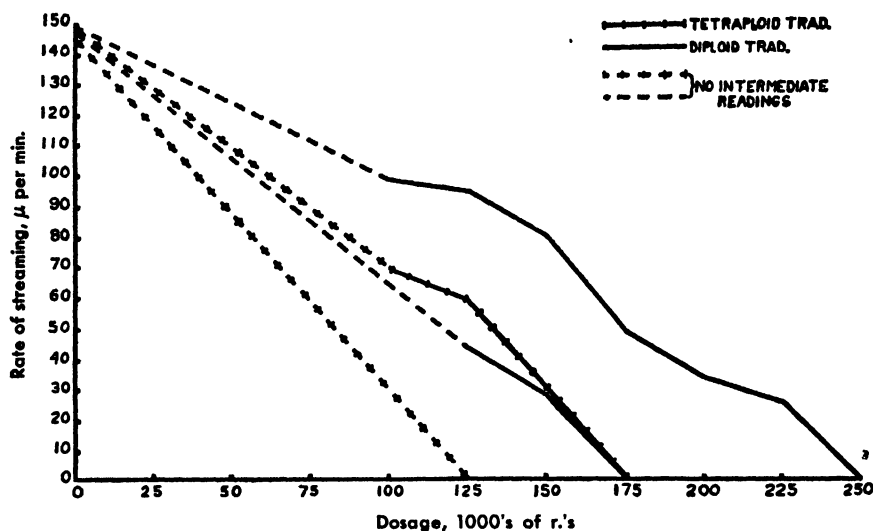


FIG. 3. The effect of varying the initial dose on cytoplasmic streaming in pollen tubes of diploid and tetraploid *Tradescantias*.

### Discussion

With few exceptions, experiments on the effects of irradiation on cells have been concerned with cell reproduction, and particularly in recent years there have been few studies based on the immediate reaction of cells to X rays. Many of the early experiments preceded the use of accurate means of measuring the amount of irradiation, and dosages were expressed mainly as a function of time and distance.

In experiments on *Paramecium* testing the immediate effects of large doses of X rays, Back and Halberstaedter (1) and Wichterman (10) have shown that these animals could survive doses up to 600,000 and 700,000 r. The recent work by Wanner (9) indicates clearly a similar tolerance of cells of *Allium* and *Hydrocharis* to X radiation. The latter work was, like our own, based to a considerable extent on the rate of cytoplasmic streaming. In Wanner's experiments no dosages were used exceeding 48,000 r., and these were not immediately lethal to the cells, although they did cause death in periods up to 50 hr. following treatment.

No significant increase in the rate of streaming was observed in any of our experiments using dosages less than 10,000 r. This is perhaps merely an indication of the great variation that exists in protoplasm. Thus, Back and Halberstaedter (1) found no visible effects on *Paramecia* motility from doses of less than 100,000 r., Wichterman (10) showed increased motility with

100,000 r., Northen and MacVicar (6) found a decrease in protoplasmic viscosity in *Spirogyra* cells, with doses of 250 to 5000 r., and Wanner (9) showed that in *Allium* and *Hydrocharis* both a decrease in viscosity and an increase of about 15% in the rate of streaming followed irradiation up to 12,000 r. Nadson and Rochlin (5) reported an acceleration of cytoplasmic movement in yeast as one of the first effects of irradiation, but they made no accurate measurement of doses used. In our cultures, variations within a single cell, particularly in the case of pollen tubes, may have been sufficient to mask any minor changes. In any case, none were observed.

Though reported by many investigators (Rochlin-Gleichgewicht (7), Williams (11 and 12), and Heilbrunn and Mazia (3)), a conspicuous change in vacuolization was not seen following irradiation of the living cells in the present experiments. Even those that were killed by the radiation seemed more homogeneous than cells that died a natural death.

With stamen hairs (Fig. 1), our data show a continuous decrease within the limits of the points of observation until the streaming ceased completely and the cell died. The extreme resistance of mature stamen hairs, which are in a permanent resting stage, to large doses (up to 750,000 r.) of X rays is of particular interest. It appears even more striking when one considers that in certain sensitive stages where cell reproduction is involved 200 r. will cause as much as 50% pollen sterility in *Tradescantia* (8).

Pollen tubes were found to be considerably more sensitive than the stamen hairs and all were "killed" by dosages of 250,000 r. or less. They are much more active than stamen hairs and represent cells in the early prophase stage. This would seem to indicate that the sensitivity of the cell may be related to the stage of the cell nucleus, but with the material used this was impossible to check.

Continuous irradiation has a more detrimental effect on the rate of cytoplasmic streaming than does irradiation administered with frequent rest periods. This is clearly illustrated in Fig. 3, where both diploid and tetraploid tubes, given an initial dose of 100,000 r., required a greater amount of irradiation to stop the streaming than did those given an initial dose of 125,000 r. The brief rest period apparently allowed some sort of recovery to take place within the cell.

Polyploidy caused no difference in effect in the case of stamen hairs, but with the pollen tubes a statistical analysis indicated a significant difference between the effects on the pollen tube of the diploid and tetraploid species, the latter showing greater sensitivity. This, of course, may represent simply a species difference rather than a change caused by an increase in chromosome number, for the two species naturally would possess genetical differences.

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## INFLUENCE OF THE AMINO ACID - DEXTROSE REACTION ON GROWTH OF *BACILLUS POLYMYXA*<sup>1</sup>

BY RUTH PETERSON, DYSON ROSE, AND L. LOEB

### Abstract

Growth of *B. polymyxa* over a 72 hr. period was not significantly affected by the brown, fluorescent materials formed by the reaction between dextrose and protein constituents of the media. During the first 24 hr. after inoculation a slight inhibition was evident in autoclaved yeast extract media, and in casein hydrolyzate media to which additional amino acid - dextrose reaction products had been added, but the inhibitory effects were never marked and disappeared within 48 hr.

### Introduction

During studies on the *Bacillus polymyxa* fermentation (2, 4) it was noted that heating the media resulted in a partial inhibition of the growth of this organism (4, 5) and this inhibition was attributed to the brown, fluorescent products formed by the reaction between reducing sugars and protein constituents of the media. Recent studies showed that products of this reaction did not inhibit growth of lactobacilli (6) and it was therefore of interest to test the assumption that these products inhibited *B. polymyxa*.

### Materials and Methods

A pure strain (N.R.C. No. C42) of *B. polymyxa* was maintained on agar slants and transferred to 10 ml. of liquid medium 24 hr. before inoculation of the experimental media. One drop of this culture was used to inoculate 10-ml. aliquots of experimental media. Cultures were maintained at 26.7° C.

This organism produces a mucoid substance that made turbidity measurements of little value for following growth. Growth was therefore followed by determining the amount of carbon dioxide produced by cultures grown in Eldredge tubes containing 5 ml. of medium and 5 ml. of 3% dextrose solution in one arm and 10 ml. of standard (approx. 0.3 N) barium hydroxide in the other. The barium hydroxide was added to the tubes just prior to inoculation; immediately afterwards the tubes were corked and sealed with paraffin. At the end of 24-, 48-, and 72-hr. growth periods, two tubes were shaken gently for 15 min. to facilitate carbon dioxide adsorption and the residual hydroxide was

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as paper No. 75 on the Industrial Utilization of Wastes and Surpluses, and as N.R.C. No. 2020.

[The October issue of Section C (Can. J. Research, C, 27: 191-268. 1949.) was issued November 7, 1949.]

titrated with standard hydrochloric acid. Control tubes containing uninoculated media were included and the carbon dioxide produced by the organism was calculated from the difference in titration between control and culture tubes. Nonproliferating cells can, of course, produce carbon dioxide, but under the conditions used in these studies the organism developed rapidly in all cultures and it seems probable that carbon dioxide production was an adequate measure of growth.

The constituents of the yeast extract and of the casein hydrolyzate media are shown in Table I. Good growth of this organism occurs over a fairly wide

TABLE I  
COMPOSITION OF THE MEDIA

Constituents	Casein hydrolyzate medium	Yeast extract medium
Yeast extract (Difco)	—	1.25 gm.
Casein hydrolyzate (Smaco)	12.5 ml.	—
Vitamin solution (3)	5.0 ml.	—
Sodium phosphate, monobasic	100 mgm.	12.5 mgm.
Sodium phosphate, dibasic	100 mgm.	12.5 mgm.
Sodium chloride	20 mgm.	2.25 mgm.
Calcium chloride	22.5 mgm.	2.5 mgm.
Zinc sulphate	2.25 mgm.	0.25 mgm.
Sodium hydroxide	To pH 7.0	To pH 7.0
Total volume	250 ml.	250 ml.

pH range (1, 4), and preliminary tests, under the conditions used in these experiments, indicated that autoclaving media of pH 7.0 did not change the pH sufficiently to affect growth of the organism.

The relative concentration of the amino acid-dextrose reaction products was estimated by determining the fluorescence of uninoculated cultures (6) using a Coleman Model 12 Photofluorometer and B-1 and PC-1 filters. The units reported are those of the instrument scale, the photofluorometer being adjusted to give a scale reading of 50.0 for a solution containing 0.200% of quinine sulphate per milliliter.

## Results

### *Influence of the Sterilization Procedure*

Carbon dioxide production by the organism was determined in media sterilized by autoclaving, at 121° C. for 13 to 15 min. with dextrose present, in media to which sterile dextrose solution was added after autoclaving, and in media sterilized by filtration through a type ST, size L6 Seitz pad. Three trials were conducted with each medium, duplicate tubes being titrated at each sampling time.

Casein hydrolyzate media had an average fluorescence of 18 to 19 when filtered or when autoclaved without dextrose. Autoclaving with dextrose increased the fluorescence to 30, 48, and 32.5 in the three trials, the variation presumably being due to minor differences in autoclaving technique.

Yeast extract contains brown, fluorescent materials and media containing it had higher fluorescence readings (44 to 46 for filtered material) than casein hydrolyzate media. Traces of reducing sugars in yeast extract eliminate much of the advantage otherwise gained by autoclaving the main supply of dextrose separately; the fluorescence of media treated in this manner was 58 to 60. Media autoclaved with all the dextrose present had a fluorescence of 66 to 68.

Fig. 1 shows the average carbon dioxide production with each medium. Only average results are given except where the differences exceeded the 5% level of statistical significance. Thus, carbon dioxide production in casein

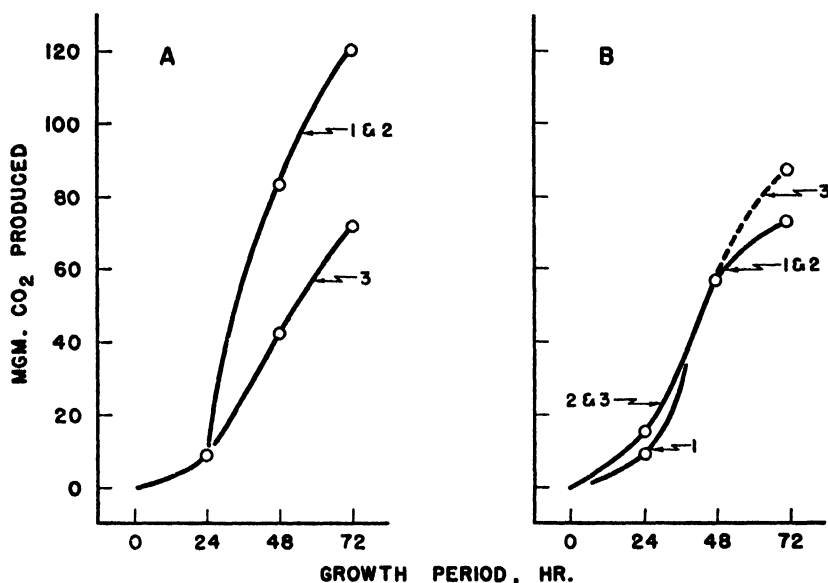


FIG. 1. Effect of method of preparation of media on growth of *B. polymyxa*. A—casein hydrolyzate media; B—yeast extract media.

Curve 1—Medium autoclaved with dextrose.

Curve 2—Dextrose autoclaved separately.

Curve 3—Filtered medium.

hydrolyzate media (Fig. 1, A), autoclaved with and without dextrose, did not differ significantly and only one line is shown. On the other hand, the amount of carbon dioxide produced on filtered media was significantly lower than on autoclaved media at the 48 and 72 hr. periods.

Results on yeast extract media were more reproducible than those on casein hydrolyzate. Carbon dioxide production in the first 24 hr. was significantly lower on media autoclaved with dextrose (Fig. 1, B) than on the other two



media but at the 48 hr. point this difference had disappeared. At 72 hr., growth on the filtered media significantly exceeded that on the autoclaved media.

#### *Influence of Added Amino Acid – Dextrose Reaction Products*

Soluble amino acid – dextrose reaction products were obtained by subjecting a mixture of casein hydrolyzate and dextrose to a temperature of 60° C. for several days. The brown solution thus obtained was filtered and suitable aliquots were mixed with 2 gm. of dextrose and made to 100 ml. These solutions were sterilized and 5 ml. added aseptically to tubes containing 5 ml. of autoclaved, dextrose-free casein hydrolyzate medium. The dextrose content of these tubes was thus 1% plus the residual dextrose of the brown solution, as compared with the 1.5% normally provided.

Triplicate trials were again made using control tubes (av. fluorescence 19) and two levels of added brown solution (av. fluorescence 91 and 203). Media at the highest fluorescence level had a transmission of about 15% of that of the controls, measured at 4200 Å.

Fig. 2 presents only the statistically significant results. At the end of the 24 hr. period, growth was inversely proportional to the fluorescence of the media, but at the 48 hr. point these differences had disappeared, and at 72 hr. the situation was reversed, greatest growth having occurred at the highest fluorescence level.

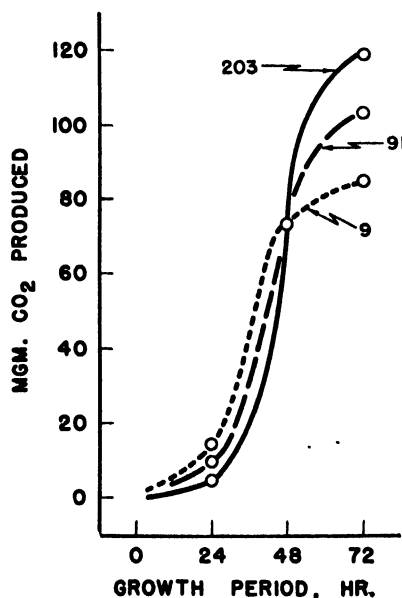


FIG. 2. Effect of added fluorescent materials on growth of *B. polymyxa*. (Numbers indicate the relative fluorescence of the media.)

#### **Discussion**

Although no direct experimental data are available, it appears probable that the Seitz filter pad adsorbed a nutrient from the casein hydrolyzate

medium and thus restricted growth of the organism. The superior growth at 72 hr. in filtered yeast extract medium may indicate a mild toxicity due to products formed during autoclaving, but the differences in fluorescence among these media are small.

Considerable unreacted dextrose remained in the solutions used in the experiments of Fig. 2, and the total carbon dioxide production in 72 hr. is undoubtedly a function of the available dextrose supply. The highest amount of fluorescent material present greatly exceeded the amounts developed by any normal autoclaving procedure; nevertheless, the organism was not only able to develop normally after the first few hours but was able to utilize the excess dextrose. It is quite evident, therefore, that any inhibition due to fluorescent materials was slight, and that the more serious inhibitions previously observed (4, 5) must have been due to some other factor such as destruction of a nutrient. This conclusion is essentially in agreement with that previously reached from studies on the lactic acid bacteria (6).

The results for the 24 hr. growth periods do indicate, however, that *B. polymyxa* is inhibited by the brown fluorescent materials to a slight extent during the early stages of growth. This is shown in both the media containing added fluorescent materials and in the yeast extract media, but small, transitory inhibitions are of little importance in fermentation studies.

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## FUNDAMENTAL STUDIES ON A SALTANT OF *HELMINTHOSPORIUM SATIVUM*<sup>1</sup>

BY NORMAN JAMES,<sup>2</sup> N. E. R. CAMPBELL,<sup>3</sup> AND CHAIM GUNNER<sup>4</sup>

### Abstract

A saltant of *H. sativum* disappeared from six different soils in which it was cultured in the laboratory within four weeks, whereas the indigenous flora persisted for nine weeks. During this nine week period numbers of bacteria showed no obvious trend, but numbers of fungi decreased. When cultured in sterilized portions of the above soils, the saltant showed a downward trend in numbers in the nine week period, a trend that was more obvious in some cultures than in others. Twenty-two of 221 isolates of fungi from soil, grain, and air, 45 of 286 isolates of *Streptomyces*, and 33 of 193 isolates of other bacteria produced evidence of antagonism to the saltant in spot inoculation studies. Filtrates prepared from two of the fungal isolates suppressed the growth of the saltant, as did also autoclaved portions of the filtrates. On the contrary, filtrates from three cultures of bacteria failed to suppress growth of the saltant, even though cultures of the bacteria did. The same 22 fungal isolates and 25 of the *Streptomyces*, when introduced separately into sterile soil along with the saltant, reduced the disease rating of barley seedlings below that produced by the saltant alone. The direct-count method of estimating numbers of spores of the saltant in a suspension was found to be reliable. Estimates based on the direct count were significantly greater than those made by the plate method. Estimates based on numbers of the saltant developing on potato dextrose agar containing small amounts of sterile soil were smaller than those made on the same medium without the soil. Even though the soil in the medium changed the pH of the medium, the difference in pH was considered not to be the primary factor in producing the lower estimates. Soil, either natural or heat sterilized, would contain the thermostable filterable substance or substances found to be produced by two species of fungi and probably produced by other species, likewise normally present in soil.

A saltant of *Helminthosporium sativum* P.K. & B., produced in 1932 by Greaney and Machacek (3), has retained the pathogenicity of the parent and remained relatively stable in cultural characteristics. However, when introduced into soil it loses its infectability. Field plots, heavily inoculated with the saltant, have produced a high incidence of disease only in the first crop (8). Since the saltant can be recognized readily on agar media, it was used in these studies to obtain information that might explain, in part at least, the varying incidence of the root rot disease of cereals (7).

### Longevity in Soil Culture

The longevity of the saltant was determined by culturing in soil under controlled conditions and making plate counts at the beginning of the culture period and at intervals for nine weeks.

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Six samples of soils ranging in texture from sandy loam to clay (moisture equivalent from 14.8 to 56.8%) were air-dried at room temperature for several weeks. Then, enough of each sample was transferred to each of 20 50-ml. Erlenmeyer flasks to give 25 gm. of moisture-free soil. Each flask was fitted with a rubber stopper, through which was inserted a 2.5 mm.-bore glass tube plugged loosely with cotton. One half of the 20 replicates of each sample were sterilized at 15 lb. steam pressure for four hours on each of two consecutive days. The other half were used in the natural state. A suspension of conidia and fragments of mycelium of the saltant was prepared from Czapek's agar cultures by washing with sterile water and mixing thoroughly. Its density was checked by the Neubauer hemocytometer method. A 2 ml. aliquot of the suspension and sufficient sterile water were added to bring each sample to its moisture equivalent. One replicate of each of the natural and the sterilized samples was used for making the initial dilutions. From these, duplicate 0.001 dilutions were used in duplicate plates for the saltant and other fungi cultured in Czapek's agar medium at 25° C. for one week; and duplicate 0.000001 dilutions for bacteria cultured in sodium albuminate agar. Each estimate thus was based on counts on two plates from each of two dilutions. The other replicates were stored at 25° C. and used at weekly intervals for making estimates on the same basis as the previous estimates.

The saltant disappeared rapidly from all samples of natural soil. In no case was it found on any of the plates after the soil cultures had been stored for four weeks. This scarcely could be attributed to loss of moisture, since the greatest loss on any sample in the nine week period was 0.52%. The indigenous flora in the samples did not show the same trend during the period of storage. In all samples the numbers of fungi became less; whereas those of bacteria fluctuated, with no obvious trend. The results on one of the samples, presented in Table I, are typical.

TABLE I

SURVIVAL OF A SALTANT OF *H. sativum* AND OF OTHER FUNGI AND BACTERIA IN SOIL SAMPLE No. 4; MOISTURE EQUIVALENT, 56.8%

Weeks	Natural soil			Sterilized soil
	Saltant ×10 <sup>3</sup>	Fungi ×10 <sup>3</sup>	Bacteria ×10 <sup>6</sup>	Saltant ×10 <sup>3</sup>
0	77.5	22.4	18.5	60.0
1	50.0	20.0	24.0	38.0
2	2.0	15.0	17.0	35.0
3	1.5	10.0	17.5	37.0
4	0.0	8.0	18.5	34.0
5	0.0	6.0	17.5	30.0
6	0.0	5.0	19.5	25.0
7	0.0	7.5	18.5	24.0
8	0.0	5.0	16.0	25.5
9	0.0	6.0	14.5	19.0

On the contrary, the saltant was isolated from all the sterilized samples on all dates, although in general, numbers showed a downward trend with time. These results are shown, along with those for raw or natural soil, in Fig. 1.

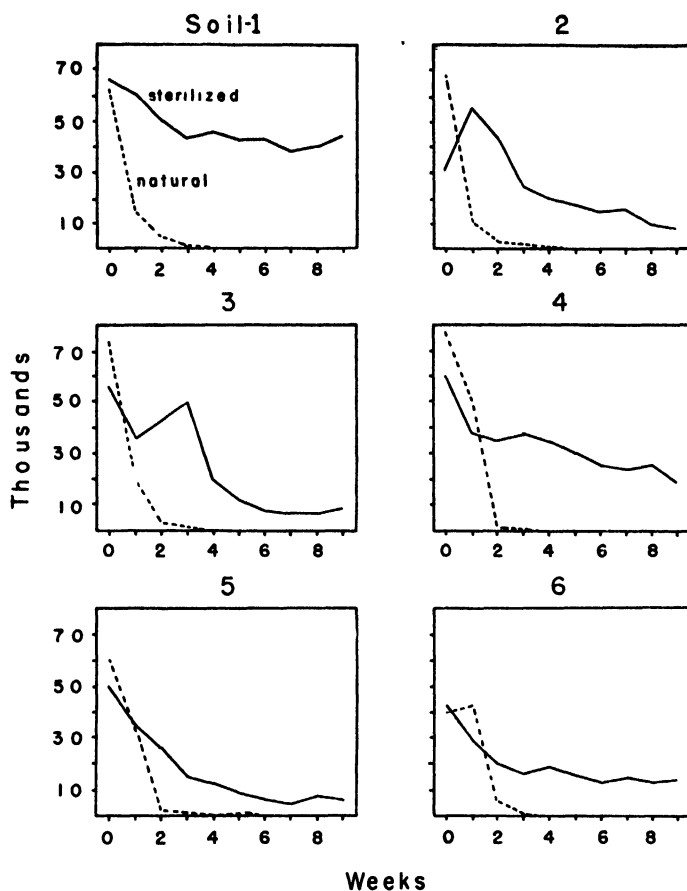


FIG. 1. Survival of a saltant of *H. salivum* in raw and sterilized soils.

### Evidence of Antagonism in Plate Culture

Each of 221 isolates of fungi, picked at random from plates prepared from soil and flour and verified as to purity by replating, was tested for antagonism to the saltant by making spot inoculations of the isolate and the saltant 1 cm. apart on Czapek's agar previously allowed to harden in a Petri plate. Triplicate pairs of inoculations were made on one plate. Incubation was at 25° C. for about 10 days. Twenty-two of these isolates showed antagonism to the saltant by slowing its growth and causing its hyphae to be turned away, or in some cases by overgrowing it and suppressing its development.

Each of 286 isolates of the *Streptomyces* Waksman and Henrici was tested similarly by the spot inoculation procedure. Forty-five of these showed evidence of antagonism.

One hundred and ninety-three isolates of other bacteria were tested for antagonism to the saltant by a variation of the above method. A loopful of a 24 hr. broth culture of the bacteria was plated in nutrient agar. After the agar had hardened, three spot inoculations of the saltant were made to the surface of the medium. Incubation was at 25° C. for 10 days. Thirty-three of these produced evidence of antagonism by causing the growth of the saltant to be less than the normal on bacteria-free plates. In order to determine whether such a dwarfing effect on a fungus could be attributed to a filterable substance produced by a nutrient broth culture of bacteria, the following experiment was carried out. Three series of dilutions varying quantitatively in numbers of cells of *Bacterium herbicola* Burri and Duggeli and of *Pseudomonas* sp., two species found to be epiphytic on the seed of cereals (6), and of a mixed culture of the flora on a sample of wheat were tested against the saltant and against *Fusarium oxysporum* (Schlecht.) Snyder and Hansen by the spot inoculation method. A second set of plates containing filtrates prepared from nutrient broth cultures of these bacteria was tested against the two fungal strains. The procedure for testing the filtrates was as follows. Potato dextrose agar containing half of the normal amount of water was added to a series of plates with (a) 5 ml. sterile water, (b) 5 ml. of a filtrate from a 10 day culture of the bacteria, (c) 5 ml. of an 0.01 dilution of the filtrate, and (d) 5 ml. of an 0.0001 dilution of the filtrate, respectively. Then spot inoculations of each fungus were made as outlined above. With each culture of bacteria and with each strain of fungus the dwarfing effect was progressively less with dilution of bacteria (smaller inoculum), whereas there was no evidence of dwarfing on plates containing any concentration of the filtrates prepared from any of the bacterial cultures. A typical result is shown in Fig. 2. There was no evidence that these bacteria produced a water-soluble filterable substance antagonistic to the saltant or to *Fusarium oxysporum*.

The above result was not obtained when filtrates prepared from two species of fungi were tested against the saltant. Five ml. of a filtrate from a 10 day culture of *Aspergillus flavus* completely inhibited growth of the saltant; 5 ml. of a 0.2 dilution suppressed growth slightly; whereas 5 ml. of a 0.01 dilution had no effect. Heat sterilized portions of the filtrate produced essentially the same results. A filtrate of *Fusarium oxysporum* likewise was antagonistic to the saltant, although sterilizing appeared to lessen the antagonistic effect slightly. A typical result is shown in Fig. 3.

### Reduction of Infection in the Presence of Antagonist

Only those isolates shown to be antagonistic to the saltant by the spot inoculation method were used for this study. The procedure was based on reports by Sallans (9) and Sanford and Cormack (10). For the first isolate, about 400 barley seeds were soaked in water for three hours, disinfected in

1 : 1000 mercuric chloride for seven minutes and rinsed five times in sterile water. One quarter of the seeds was transferred aseptically to a 60 ml. suspension of the saltant in sterile water; another quarter to a suspension of saltant and isolate; another to a suspension of the isolate; and the fourth to sterile water. The last two served as controls. After one hour the seeds were removed from the liquid (merely by inverting the bottle carefully) and incubated at 25° C. for 20 hr. Twenty seeds from each suspension were planted in each of two 1 liter flasks containing 300 gm. of a sterile, moist 3 : 1 soil-sand mixture. After 10 days, seedlings were rated for infection by the method of Greaney, Machacek, and Johnson (4). The other isolates were investigated in the same manner, except for the omission of the suspension of the saltant, on which the disease rating had been established. In every case seeds treated in the suspension of the isolate alone, and in sterile water, produced normal seedlings whereas those treated in a suspension of the saltant and a fungal isolate produced seedlings with a disease rating smaller than that produced by seeds treated in a suspension of the saltant alone. The results on the fungal isolates are shown in Table II. Likewise, 25 of the 45 isolates of the genus *Streptomyces* reduced the disease rating significantly below that of the saltant alone.

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FIG. 2 Effect of a filtrate from a portion of, and of a culture of *Pseudomonas* sp., on development of a saltant of *H. sativum*.

Above—left to right:

Plate 1.—Saltant on double concentration potato dextrose agar containing 5 ml. sterile water.

Plate 2.—Saltant on the agar containing 5 ml. of an 0.0001 dilution of a filtrate from a 10-day culture of the bacteria.

Plate 3.—Saltant on the agar containing 5 ml. of an 0.01 dilution.

Plate 4.—Saltant on the agar containing 5 ml. of the filtrate.

Below—left to right:

Plate 1.—Same as Plate 1 above.

Plate 2.—Saltant on the agar freshly inoculated with 1 ml. of an 0.01 dilution of a culture of the bacteria.

Plate 3.—Saltant on the agar with 1 ml. of an 0.1 dilution.

Plate 4.—Saltant on the agar with 1 ml. of the broth culture.

FIG. 3. Antibiotic effect of filtrate from 10 day culture of *Aspergillus flavus* on development of a saltant of *H. sativum* on double concentration potato dextrose agar.

Above—natural filtrate of *A. flavus* cultured in potato dextrose liquid medium.

Below—sterilized filtrate.

Left to right:

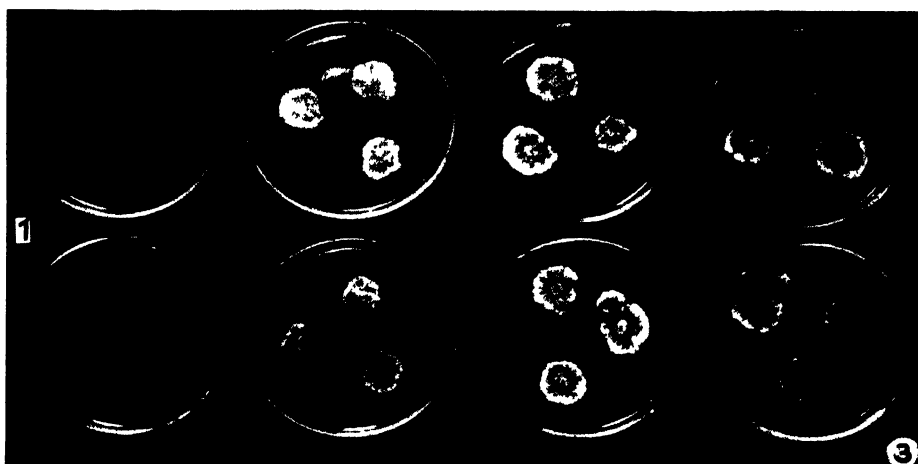
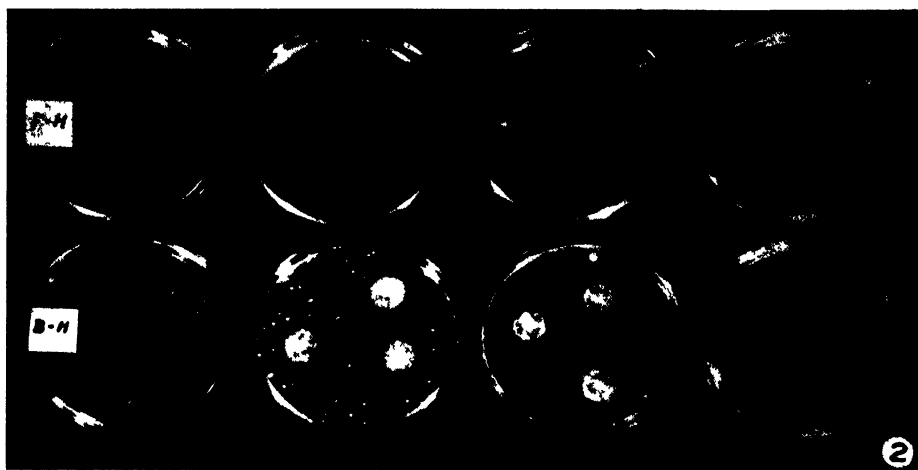
Plate 1.—Five ml. of filtrate.

Plate 2.—Five ml. of 0.2 dilution of filtrate.

Plate 3.—Five ml. of 0.01 dilution of filtrate.

Plate 4.—Five ml. of sterile water.

PLATE I



FIGS. 2-3.





TABLE II

EFFECT OF ISOLATES FOUND TO SHOW ANTAGONISM TO A SALTANT OF *H. sativum* IN PLATE CULTURE ON INFECTION OF BARLEY SEEDLINGS BY THE SALTANT

Isolate	Mean disease rating	
	Saltant and isolate	Difference from saltant*
<i>Aspergillus candidus</i> Link	23.5	-17.8
<i>A. flavipes</i> (Bain. and Sart.) Thom and Church	2.0	-39.3
<i>A. flavus</i> Link		
Isolate 1	1.0	-40.3
Isolate 2	10.5	-30.8
<i>A. glaucus</i> Link	20.0	-21.3
<i>A. nidulans</i> (Eidam) Winter	1.0	-40.3
<i>Cladosporium herbarum</i> (Persoon) Link	6.5	-30.8
<i>Fusarium oxysporum</i> (Schlecht.) Snyder and Hansen		
Isolate 1	1.0	-40.3
Isolate 2	1.0	-40.3
<i>Mucor varians</i> Povah	6.0	-35.3
<i>Penicillium canescens</i> Sopp	7.5	-33.8
<i>P. cesiae</i> Bainier and Sartory	3.5	-37.8
<i>P. commune</i> Thom	15.0	-26.3
<i>P. corylophylum</i> Dierckx		
Isolate 1	2.5	-38.8
Isolate 2	5.5	-35.8
<i>P. hagemi</i> Zaleski	18.0	-23.3
<i>P. krzemieniewskii</i> Zaleski	8.5	-32.8
<i>P. lilacinum</i> Thom	5.5	-35.8
<i>P. nigricans</i> Bainier-Thom	15.5	-25.8
<i>P. pfefferianum</i> (Wehmer) Westling	7.5	-33.8
<i>P. purpurogenum</i> Stoll	5.5	-35.8
<i>P. roquefortii</i> Thom		
Isolate 1	7.5	-33.8
Isolate 2	5.0	-36.3
<i>P. roseo-maculatum</i> Biourge	**	
<i>P. steckii</i> Zaleski	13.5	-27.8
<i>P. terrestre</i> Jensen	**	
<i>Scopulariopsis brevicaulis</i> (Sacc.) Bainier	1.0	-40.3
<i>Trichoderma lignorum</i> (Tode) Harz	3.0	-38.3

\* Necessary difference at 0.05 = 13.2 and at 0.01 = 17.5.

\*\* Variance exceeded control limits established at the 3 sigma level (1).

### Estimating Numbers of the Saltant

While the principle of estimating fungal populations by the plate-count method is open to criticism, the fact holds that the method is used in relation to many different problems. It was used in this study for comparison with the direct-count method.

A Neubauer haemocytometer slide, employed in the routine quantitative determination of spores and fragments of mycelium of molds in certain foods, was used for the direct counts. The reliability (5) of an estimate based on this procedure was established, as follows. A suspension of spores and fragments of mycelium in sterile water was made from a 14 day *zapek's* agar culture of the saltant. Counts of spores were made on five fields, each representing 0.0001 ml. of the suspension, and averaged. This was replicated

four times on one suspension. A Chi square value was calculated from the average counts on the four slides from one suspension, an additional degree of freedom being lost due to the use of average counts (2). This procedure was carried out on 96 different suspensions. The goodness-of-fit test (2) on the distribution of Chi square values obtained thus yielded a  $P$  value of 0.13. The results are shown in Fig. 4.

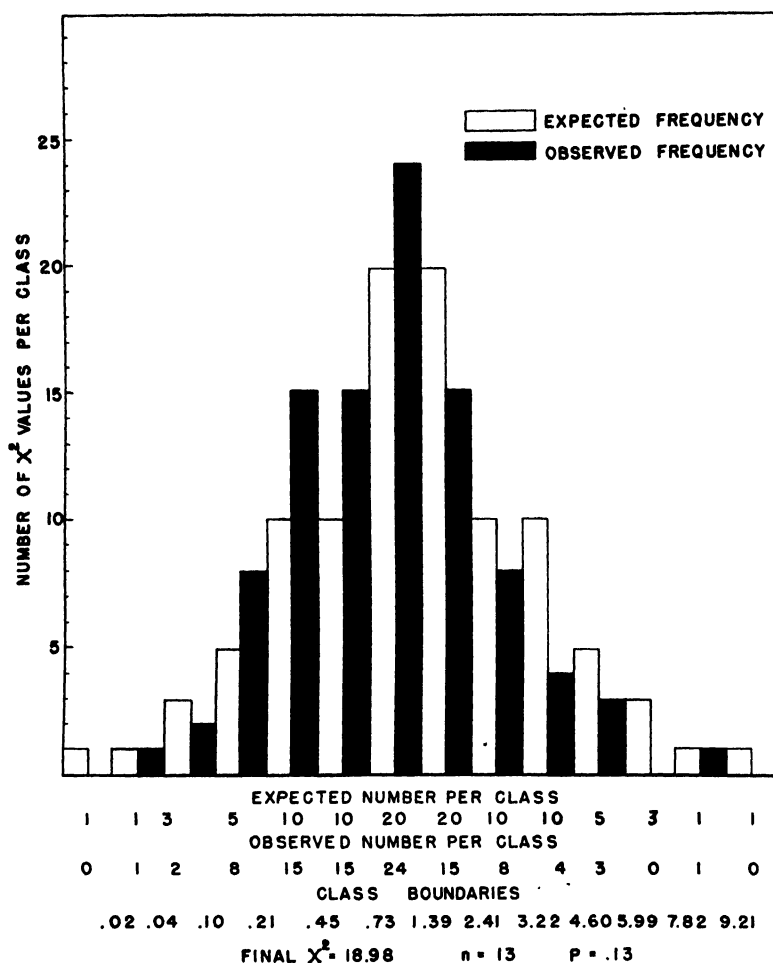


FIG. 4. Distribution of Chi square values calculated from direct counts of spores in 96 suspensions of the saltant.

A study of the relationship between estimates made by the direct-count and the plate-count methods was carried out on a second set of 96 suspensions. Each estimate by the direct-count method was based on counts on five fields from each of four replicate slides prepared from one suspension and, by the plate-count method, on four replicate plates prepared from an 0.0002 dilution of the same suspension cultured in Czapek's agar, adjusted to pH 4.5, at

25° C. for seven days. Two estimates by the direct-count method were made on each suspension: (a) spores and (b) spores, plus fragments of mycelium. The latter estimates, obviously being larger on each suspension, were not considered further. Estimates by the plate-count method were smaller than those based on direct counts of spores on 92 of the 96 suspensions studied; and on the remaining four the difference was small. The difference between means of the 96 estimates made by the two methods was 80,340 per ml. The necessary difference (2) for significance at the 0.01 level was 44,320.

### Effect of Soil in Medium on Estimates of the Saltant

The suspensions used in the studies referred to above were water suspensions. They lacked the soil particles and solutes that would be present when estimating a soil population. The following experiment was carried out to study the effect of sterile soil on estimates of the saltant based upon plate counts. Two gm. soil was sterilized with sufficient water to make the 90 ml. blank to be used for preparing the 0.0002 dilution of the suspension to be plated. The 1 ml. placed in each plate thus contained 0.02 gm. soil—or 100 times as much as would be present in plates prepared from soil at this dilution. The larger amount was used merely to make any effect more readily detectable. The experiment was replicated eight times on each of 12 different soils. It was carried out at the same time and with the same suspensions as were used in the experiment dealing with comparison of estimates by the direct-count and plate-count methods. The plate-count estimates on the water suspensions of the saltant thus served as the controls for this study.

In all of the 96 suspensions tested, estimates made from plates containing sterile soil were smaller than those from plates without soil. The analysis of variance follows:

Source	D.f.	Mean square	F	F at 0.05
Soils	11	63,332.27	50.32	1.90
Treatments $\times$ soils	12	9131.34	7.25	1.87
Replicates $\times$ soils	84	24,243.03	17.67	1.87
Error	84	1258.66		

The effect for the interaction of replicates and soils was expected, since the experiment was carried out with different cultures of the saltant and it was not feasible to prepare suspensions with the same number of spores.

### Effect of Soil in Medium on pH of Medium

The following experiment was carried out to determine the effect of soil in the medium on pH of the medium. The pH of a batch of Czapek's medium

was determined. Then 0.02 gm. and 0.10 gm., respectively, of each of the 12 soils, sterilized in water suspension, were added to 10 ml. Czapek's liquid medium and the pH determined. The results, presented in Table III, show

TABLE III  
EFFECT OF STERILE SOIL IN CZAPEK'S LIQUID MEDIUM ON pH OF THE MEDIUM

Soil	Control		Soil per 10 ml. medium			
	1	2	0.02		0.10	
			1	2	1	2
a	4.50	4.50	4.80	4.70	5.10	5.10
b			4.90	4.95	5.50	5.50
c			4.80	4.80	5.00	4.95
d			5.60	5.60	6.90	7.00
e			4.70	4.75	5.00	5.00
f			4.80	4.80	5.10	5.05
g			4.65	4.60	5.10	5.00
h			4.75	4.75	4.90	4.95
i			4.70	4.65	5.00	5.00
j			4.70	4.70	4.85	4.80
k			4.75	4.80	5.00	4.90

that with every soil the pH of the medium containing 0.02 gm. of soil was higher than that of the control and the pH of the medium containing 0.1 gm. soil was higher than that containing 0.02 gm. soil. It should be noted that the 0.02 gm. soil per 10 ml. was equivalent to the 0.02 gm. per plate used in the experiment reported immediately above. It is scarcely probable that the small difference in pH was the only factor responsible for the significantly lower estimates from plates containing soil in the medium.

### Discussion

It appears obvious that the reduction in infection under field conditions was due to the destruction of this saltant in soil; and not merely to loss of infectability. In the laboratory experiments the destruction was greatest in natural soils—the saltant not being found after four weeks in any of the samples. But the effect was evident in heat sterilized soil—numbers of the saltant in sterilized soil showing a downward trend with time and numbers of the saltant that developed on Czapek's agar containing small amounts of soil being fewer than on the same medium without the soil. This would indicate that the destructive and inhibiting substance or substances probably was a product of the metabolism of the indigenous soil flora. It was produced in larger amounts in some soils than in others. Some was thermostable in soil. Even the relatively extreme sterilizing exposure did not inactivate all. The experiments on antagonism showed that it was produced by a number of isolates of fungi, *Streptomyces*, and bacteria (probably). It was filterable.

The spot inoculation method used for measuring antagonism showed a fair degree of agreement with the reduction-in-disease-rating method. However, correlation studies were not carried out since the data were not considered appropriate. The rating for antagonism was at three arbitrary levels only; whereas that for reduction of infection was at levels of 0.1 up to 41.3. The latter was the disease rating of the saltant in this study.

The finding that the bacterial isolates from wheat did not produce a filterable substance in sufficient quantity to be antagonistic to the saltant, or to *Fusarium oxysporum*, may be accepted as suggesting that the suppression of *Helminthosporium sativum* by the epiphytic bacteria on wheat seeds, reported by Simmonds (11), probably was due primarily to competition for nutrients in the medium. Preliminary incubation of moistened seed would produce a numerically increased active epiphytic flora, which would compete for nutrients in the medium surrounding the seeds. The effect would simulate that of antagonism.

### Acknowledgments

The authors are pleased to express thanks to Dr. J. E. Machacek, Plant Pathologist, Dominion Laboratory of Plant Pathology, Winnipeg, for his helpful interest in these studies and for checking the identity of the fungi listed, and to Associate Professor G. B. Oakland, Department of Actuarial Science of this university, for his advice on the statistical aspects of the investigations.

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# A STUDY OF SUGAR BEET ROOTROT IN SOUTHERN ONTARIO<sup>1</sup>

BY W. E. McKEEN<sup>2</sup>

## Abstract

In southern Ontario rootrot of sugar beets may be caused by *Aphanomyces cochlioides* Drechs., *Pythium aphanidermatum* (Edson) Fitz., *Pythium ultimum* Trow, or *Rhizoctonia solani* Kuehn. The distribution of these fungi was found to be limited to soil type rather than to locality, *A. cochlioides* thus being found in the clay soils and *P. aphanidermatum* only in the sand loam. They were not present in the subsoil and were most abundant in the top two or three inches of surface soil. *A. cochlioides*, which was found for the first time in Canada in 1946, causes the so-called blackroot of sugar beets, and is the most economically important pathogen encountered. Blackroot appears either as an early acute attack or a later chronic one, but the acute phase is of major importance and occurs in epiphytotic during seedling development, usually when the beet is from two to five weeks old. Weather records and experiments have shown that serious disease outbreaks only occur when moisture is abundant and soil temperature exceeds 60° F. Susceptibility is sharply correlated with the developmental rhythm of the seedling and ends when the cortex of the hypocotyl is sloughed off. *A. cochlioides* is homothallic, attacks the hypocotyl of the beet, and develops in the intercellular spaces of the cortex. It will not grow at relative humidities of less than 99% and this sensitivity to all but extremely high humidity is believed to limit its parasitic activity. Microbiological studies indicated that the number of organisms in the rhizosphere of diseased sugar beet roots is much larger than in that of healthy beet roots. Since parallel rhizosphere responses followed local artificial killing of roots, they are believed to be due in part at least to the utilization of necrotic tissues or substances released on their breakdown. Streptomycin, but not penicillin, was effective in controlling rootrot in greenhouse experiments. Control with Arasan is achieved in part through a direct fungicidal action, but the evidence suggests that it may be due to a shift in the microbiological balance of the soil flora unfavorable to the pathogen.

## Introduction

In Ontario, sugar beets are grown most extensively in the southwestern part of the province, the largest acreages occurring in Kent, Lambton, Middlesex, and Elgin counties, with much smaller plantings in Huron, Perth, and Essex. While most of the beets are produced on heavy Brookston and Clyde clay loam soils, some excellent crops are found on light sandy loam in the vicinity of Exeter in Huron, and tremendous yields are harvested from the marl soil of the Blackwell area in Lambton.

During the last 15 years, in the area indicated, about 27,000 acres of beets with an average yield of approximately nine tons per acre have been harvested annually. All of these beets have been marketed through the one channel, the Canada and Dominion Sugar Company, which has produced an average of 70,000,000 lb. of sugar, 11,100 tons of molasses, and 13,700 tons of dried beet pulp each year. In a single year the growers have received as much as

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four million dollars for the crop and, in addition, they have beet crowns and tops as green feed for livestock. The factory workmen, transportation agencies, and supply companies receive another two and a half million dollars annually so that, everything considered, the sugar beet industry has reached sizeable proportions in Ontario.

Although the sugar beet may suffer from many fungus, virus, and nematode diseases, most of these, up to the present time, have proved to be of little economic significance in Ontario. *Cercospora* leaf spot, which was once a devastating disease, has been completely controlled by the use of resistant varieties. At the present time rootrot is the most widespread and economically significant pathological condition with which the grower has to cope. It is primarily a seedling disease and is the major factor responsible for poor stands of beets. Faced with this uncertainty of obtaining and maintaining a good stand of plants and the possibility of losing an entire crop, the farmer is reluctant to plant his fields to beets, and an adequate acreage is difficult to maintain. The mechanization of the sugar beet industry, moreover, has made more urgent the need for controlling this disease, since the machines used for blocking and thinning operations operate on the basis of a practically perfect seedling stand.

In 1944 the Canada and Dominion Sugar Company reported that 685 acres, valued at approximately 90,000 dollars, were completely wiped out by rootrot and in many other fields various percentages of the seedlings were killed and very spotty stands resulted. In 1940 a still greater destruction was caused and every year the disease is present throughout the whole beet growing district. With continued production of beets it has tended to become more serious and, because of it, many growers no longer attempt to grow beets. In fact, rootrot was probably the major factor responsible for the abandoning of beet growing in Essex County.

The present study was undertaken to determine the identity and relative importance of the causal organisms responsible for root degeneration in sugar beets and to study the factors influencing the severity of such diseases as might be found in Ontario.

### Survey of Pathogens Associated with Rootrot

To determine the organisms responsible for root decay, seedlings that showed the first symptoms of rootrot were removed from the soil and washed thoroughly in running water before they were placed in Petri plates containing agar media or water. Surface sterilization with mercuric chloride or alcohol was only necessary when the infected tissue was placed on the agar media. Most of the diseased seedlings from which isolations were made were obtained from commercial fields throughout the sugar beet district but additional isolations were made from sugar beet seedlings that had grown in infested soil in the greenhouse. The following fungi were obtained: *Aphanomyces cochlioides* Drechs., *Pythium aphanidermatum* (Edson) Fitz., *Pythium ultimum* Trow, *Rhizoctonia solani* Kuehn, *Fusarium* spp., *Penicillium* spp., *Actinomyces*



spp., *Mucor* spp., and some that were not identified. Frequently only one fungus emerged from the diseased seedling when it was placed in water. Pathogenicity tests were conducted by growing sugar beet seedlings in sterile soil inoculated with cornmeal and cultures of all the above organisms. In these tests only *A. cochlioides*, *P. aphanidermatum*, *P. ultimum*, and *R. solani* proved to be pathogenic.

The distribution of the pathogens was found to be related to soil type rather than to locality, as can be observed in Table I. *A. cochlioides* was

TABLE I

THE FUNGI ISOLATED AND THEIR RELATIVE FREQUENCY OF ISOLATION FROM SUGAR BEET SEEDLINGS GROWN IN FOUR SOIL TYPES CHARACTERISTIC OF THE AREAS OF COMMERCIAL BEET PRODUCTION

	Isolations				
	Total	Brookston clay	Clyde clay	Exeter sandy loam	Blackwell marl
<i>A. cochlioides</i>	164	137	27	0	0
<i>P. ultimum</i>	102	88	8	0	6
<i>P. aphanidermatum</i>	32	0	0	32	0
<i>R. solani</i>	18	15	2	0	1
<i>Fusarium</i> spp.	22	6	0	0	16
<i>Penicillium</i> spp.	5	3	0	2	0
<i>Actinomyces</i> spp.	6	4	2	0	0
<i>Mucor</i> spp.	7	5	0	1	1
Unidentified spp.	17	11	2	1	3

found only in the clay soils, whereas *P. aphanidermatum* was the only pathogen in the sandy loam of the Exeter district and was not found in any of the other soil types. *P. ultimum* and *R. solani* were present in all but the Exeter sandy loam. It was observed that in the Blackwell marl soil where rootrot was of no significance neither *A. cochlioides* nor *P. aphanidermatum* was present. Judging from the relative frequency of isolation and the disease symptoms observed in the field, *A. cochlioides* appeared to be the most virulent and economically important of the pathogens and for this reason was studied in some detail and is reported on first.

### Blackroot Caused by *Aphanomyces cochlioides*

#### Symptoms

*A. cochlioides*, frequently designated as the water mold, causes what is commonly called blackroot of sugar beets, which is the most dreaded of the rootrot diseases. Blackroot appears either as an early acute attack or a later chronic one, but it is the acute phase that is of major importance and exhibited in epiphytotics during seedling development, usually when the beet is from two to five weeks old.

The symptoms in very young seedlings are not significantly different from those of seedlings attacked by *Pythium*, except that they assume a darker color, but those produced after true leaves are formed are striking and characteristic. A necrosis of the hypocotyl begins at the ground level, spreads rapidly to the cotyledonary leaves, and give the hypocotyl a characteristic shiny jet-black color that makes it appear as though scorched by a flame. This, no doubt, gave rise to the term blackroot, although this is somewhat of a misnomer and "black hypocotyl" would be more accurate.

In a short time the blackened cortex of the hypocotyl dries; the stem shrivels to a mere thread, and only the vascular system remains. The seedling topples over, withers, and is frequently broken by the wind. Fig. 1 shows seedlings with these characteristic symptoms. Whole fields of sugar beet seedlings may be destroyed within three or four days if the weather is moist and warm. If lower temperatures of around 50° F. prevail, infected seedlings may live for at least two weeks even in saturated soil. The leaves at first have a bluish tint and later slowly turn yellow as the fungus moves up the stem. If the soil dries and the temperature remains low, new lateral roots (Fig. 2) may be produced and the seedling recovers. The grower calls this 'coming out' of blackroot.

After the seedlings have produced three to five pairs of true leaves, the cortex of the hypocotyl fissures (Fig. 3) and this tissue is sloughed off owing to the formation of a periderm below. In the root the cortex drops off much earlier, usually when the seedling is about two or three weeks old. After the cortex is lost the seedling no longer suffers from the acute phase of attack.

In July and August the chronic phase of the disease may occur on the larger beets, the infected individuals usually being scattered sparsely throughout a 'blackroot' field. The affected plants show a wilting that becomes more severe from day to day and finally the leaves begin to die. When these plants are pulled out of the soil, infection will usually be found to have occurred in the extreme tip of the tap root and this necrotic tip is often embedded in wet soil. A slight amount of infection in this tip region may cause pronounced wilting. Three beets attacked in this manner are shown in Fig. 4. Very infrequently a beet may be invaded in the middle of the tap root (Fig. 5) in which case the lesion extends completely around the root and causes considerable constriction.

#### *Isolation of Aphanomyces cochlioides*

The generic name '*Aphanomyces*' means 'illusive fungus' and no better term could be applied to this pathogen. It cannot be isolated by ordinary laboratory techniques, only bacteria and other secondary organisms being obtained when pieces of seedling tissue, infected with *A. cochlioides*, are surface sterilized and plated out on agar media. If, however, portions of the infected seedlings are thoroughly washed and placed in Petri dishes containing 15 to 20 cc. of sterile water, after a few hours at room temperature *A. cochlioides* can be

detected by the thousands of evacuation tubes that it sends out and by the groups of encysted zoospores (Fig. 6). Zoosporangial formation and zoospore discharge can be readily observed in this water medium.

If a pure culture is desired, a Van Tieghem cell is placed in the center of a deep Petri dish immediately after a thick layer of warm 3% agar medium has been poured into it. The Van Tieghem cell has three little wires or pins clamped around it to raise it from the bottom of the Petri plate. Then a piece of tissue with some mycelial growth is rinsed in sterile water and pushed well down into the agar within the cell. The hard medium inhibits most bacteria from growing through it, and they develop only on the surface of the agar inside the Van Tieghem cell. The water mold, however, grows down through the medium, and emerges on the outside of the Van Tieghem cell free of bacteria and thus can readily be isolated in a pure state.

### *Life History of Aphanomyces cochlioides*

Asexual reproduction can be induced without difficulty by immersing, in water, seedlings that are infected with *A. cochlioides* or young mycelia on a thin layer of cornmeal agar. After three or four hours hyphae can be seen radiating out from the infected tissue, and a little later, among them, tiny white specks, which are groups of encysted zoospores, can be observed with the naked eye. Under the microscope, short, predominantly unbranched, more or less sinuous threads will be found to project from the host. These efferent threads, which are evacuation tubes, are nonseptate, full of dense protoplasm, and from 8 to 10  $\mu$  in diameter and up to 2 mm. in length.

The protoplasm in the evacuation tubes and in the threadlike sporangia that are within the host, now breaks up into little rods about 12  $\mu$  long (Fig. 7). The first evidence of spore formation is the appearance of narrow transverse transparent bands across the protoplasm at regular intervals in these tubes. About five minutes later the sporangiospores flow rapidly from these tubes and sporangia and instantaneously round up, each spore surrounding itself with a thin cellulose membrane. Some sporangiospores vary from 6 to 15  $\mu$ , but are usually 8 to 9  $\mu$  in diameter and as many as 300 may aggregate at the end of a tube where, as Figs. 6 and 8 show, they look like bunches of grapes. A single group is shown in Fig. 9.

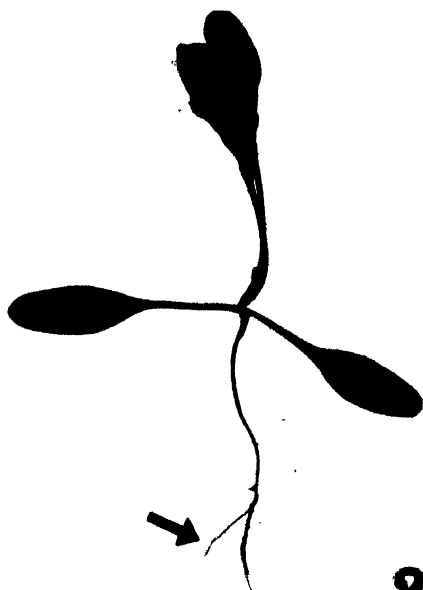
After three or four hours a small elevation is formed on each spore, and this gradually increases until within three to five minutes a papilla about 2  $\mu$  in diameter is produced. Then the membrane of this papilla breaks at its apex, and the protoplasm flows out rapidly through the necklike opening and forms

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FIG. 1. Seedlings showing acute symptoms of blackroot caused by *A. cochlioides*. From left to right: healthy; base of hypocotyl blackened; hypocotyl completely black; cortex completely destroyed and only vascular strand remaining. FIG. 2. A seedling recovering from blackroot; observe the new lateral root. FIG. 3. The cortex of the hypocotyl of the healthy beets has fissured and is beginning to slough off. The seedlings have three or four pairs of true leaves. FIG. 4. Beets suffering from the chronic phase of attack of *A. cochlioides*. Note that the root tips are necrotic. FIG. 5. A girdled beet, another manifestation of the chronic phase of blackroot.



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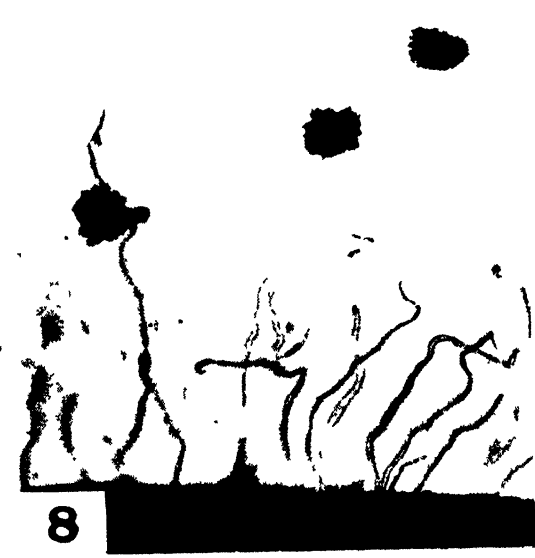
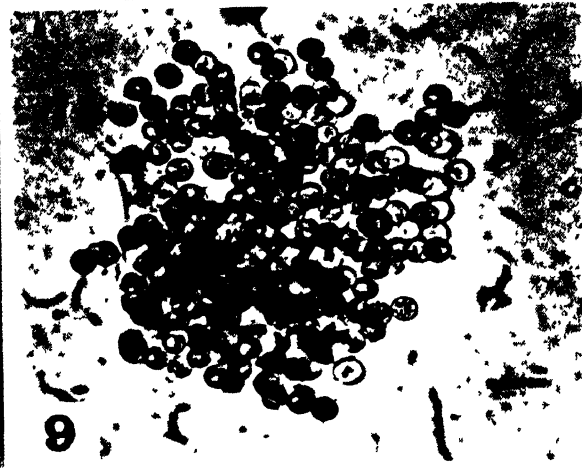
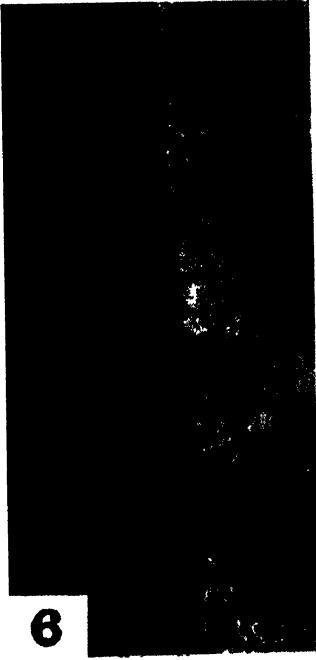
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another sphere, the cylindrical wall of the papilla persisting on the empty cyst membrane. Almost immediately two flagella, about  $24\ \mu$  long, inserted in a slight depression of the spore, are detected waving in the water. The zoospores gradually increase their oscillating motion and the lashing of their flagella becomes more vigorous until after about 10 to 20 min. they are extremely active and tear away from the papilla membranes. The zoospores are usually  $13\ \mu$  long and 7 to  $8\ \mu$  in diameter, but large double spores have been observed. A whole group of encysted zoospores may be emptied in four or five hours. The zoospores swim vigorously for three or four hours, and then settle down and produce a germ tube, as is shown in Fig. 10. In distilled water the tubes may measure 2 mm. in length and occasionally they produce several branches some of which may reach a length of  $70\ \mu$ .

The writer estimated that on a piece of infected hypocotyl 1 in. long and  $1/16$ th in. in diameter as many as 500 evacuation tubes might be present, each of which would discharge on the average 100 zoospores. Thus from 0.003 cu. in. of infected tissue, 50,000 zoospores might be produced and liberated within one day. Each of these spores may attack a healthy seedling, and produce zoospores again within a couple of days, so that under favorable conditions the asexual stage may rapidly produce very abundant inoculum.

After zoospore production ceases, the sexual organs make their appearance, and finally are found scattered rather liberally throughout large portions of the collapsed host as is seen in Fig. 11. The oogonia (Fig. 12) are subspherical, smooth, usually 20 to  $29\ \mu$  in diameter and provided with a wall of somewhat irregular inner contour about  $1.6\ \mu$  in thickness. From one to four antheridia (Fig. 13), 6 to  $10\ \mu$  in diameter and 10 to  $18\ \mu$  in length, become wrapped about individual oogonia. The antheridia (Fig. 14) are club-shaped, separated from the stalk by a septum, and frequently are partly countersunk into an abrupt depression in the oogonial wall. In this region a short fertilization tube develops. The diameter of the oospore is about  $5\ \mu$  less than that of the oogonium, and each contains an eccentrically placed reserve globule about  $12\ \mu$  in diameter. The oospores are resistant to unfavorable climatic conditions and remain viable for a long time. They are shown to survive for one but not two years in air-dried, naturally-infested soil.

To find out whether *A. cochlioides* was homo- or heterothallic, a pure culture growing on cornmeal agar was placed in sterile water in a Petri dish. Zoospores that had encysted after they emerged from the sporangia were scattered over an agar plate from which individual ones were picked up by means of a biscuit cutter and transferred to another plate flooded with water.

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FIG. 6. *A. cochlioides* growing out from infected tissue, with groups of encysted zoospores at the tips of efferent tubes. FIG. 7. Numerous efferent tubes of *A. cochlioides* radiating out from the infected tissue. They are filled with dense protoplasm, which, in some, has broken up into rods or zoospores. FIG. 8. Groups of zoospores encysted at the tips of three efferent tubes and in one tube four zoospores have not escaped. FIG. 9. A single group of encysted zoospores at the tip of an evacuation tube. FIG. 10. A zoospore germinating after secondary encystment.

In every case the colonies resulting from individual zoospores produced normal sexual fruiting bodies. *A. cochlioides* is accordingly homothallic, unless perchance within the species, strains of different potentialities exist.

### *Host-Parasite Relations*

From freehand sections of living material the development of the fungus has been traced in the seedling. Mycelium has been found only in the intercellular spaces of the cortical tissue of the hypocotyl and root before the cortex is sloughed off (Fig. 15). The hyphae are rather scanty in affected tissues and a relatively small portion of the intercellular spaces is occupied. *A. cochlioides* spreads slightly in advance of visible symptoms and may migrate up to the hypocotyl into the cotyledonary leaves. The cell walls adjacent to the mycelium assume a dark brown to black color and, when stained with cotton blue, have a greenish yellow appearance. Sometimes in the later stages of infections considerable amount of blackish granular material may surround the hyphae in the intercellular spaces.

Observational evidence indicated that the pathogen probably entered through the hypocotyl since necrosis consistently appeared first on its lower part. To test this, seedlings were transplanted to 4-in test tubes into which two small holes had been blown, one in the bottom and the other one about one inch and a half up the side. Through these air and water could enter freely. Sterile soil was then placed around the root and a 50% mixture of vaseline and low-melting paraffin was poured around the seedling so that the top of the root and the bottom of the hypocotyl was sealed off (Fig. 16). Inoculum, in the form of pieces of infected seedlings or bits of agar containing *A. cochlioides*, was placed sometimes below and sometimes above the paraffin-vaseline seal; other seedlings were left uninoculated as checks. About one-half inch of water was then poured around the hypocotyl and the test tubes were placed in a beaker that contained about an inch of water.

In all test tubes in which the inoculum was introduced above the paraffin-vaseline seal, typical blackroot symptoms of the beets occurred and the plants soon died as a result of infection by *A. cochlioides*. When inoculum was introduced below the seal, blackroot symptoms did not appear within three weeks, at which time the experiment was discontinued. Of the 87 plants that had inoculum introduced with the roots, 16 died soon after transplanting, but from none of them could *A. cochlioides* be isolated. However, approximately the same percentage of check plants also died immediately after transplanting, owing, apparently, to some mechanical injury or the use of a paraffin-vaseline mixture that was too warm. Thus it appears that penetration is limited to and takes place readily through the hypocotyl. There are numerous open stomata on the hypocotyl of the sugar beet and these may well be the portal of entry for *A. cochlioides* since the hypocotyl has a thick cuticle and since the pathogen always appears to be intercellular

In greenhouse experiments the chronic phase has never been observed on beets growing either in naturally- or artificially-infested soil, even when the soil

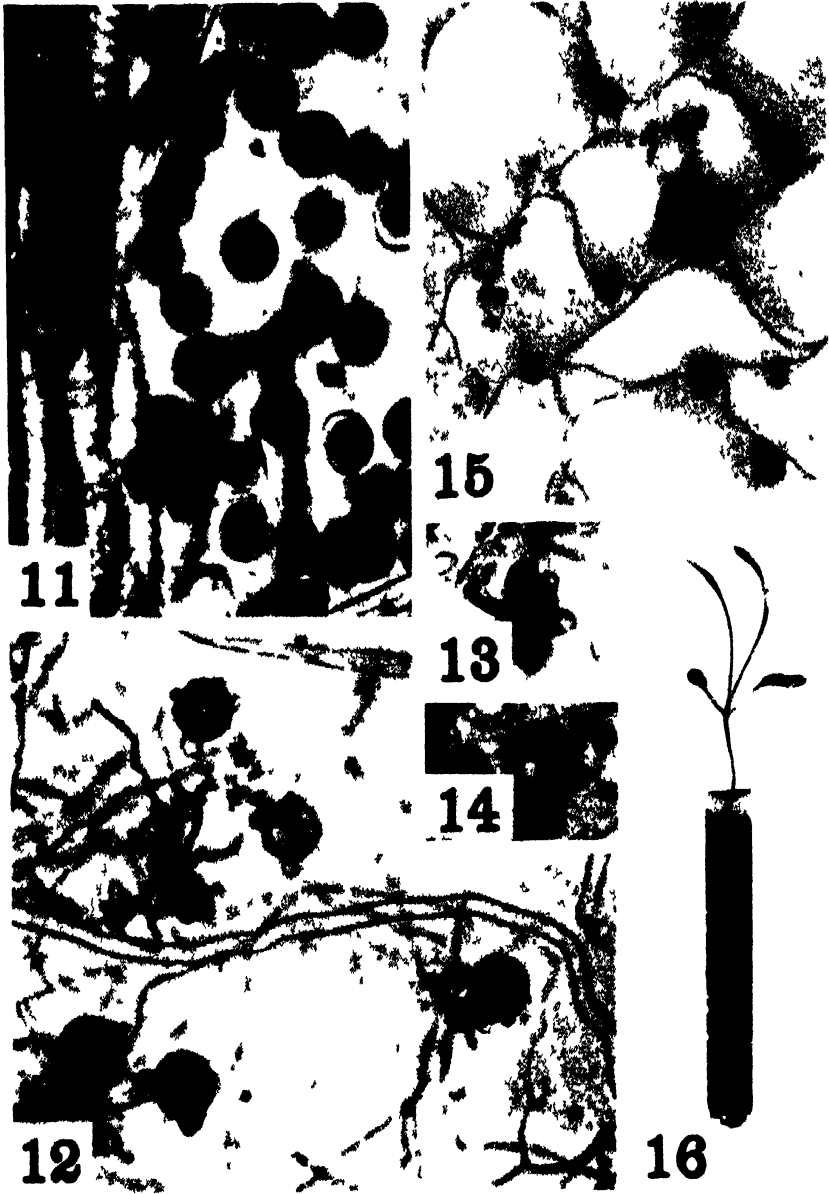


FIG. 11—A group of oögonia of *Cochlioides* in the cortex of the infected beet. FIG. 12—Oögonia at various stages of development: lower left oögonium prior to oospore formation, immediately to right, oögonium with oospore, at right an oögonium within which there is an oospore with an eccentrically placed reserve globule. FIG. 13—Oögonium with four antheridia attached. FIG. 14—A club shaped antheridium separated from the stalk by a septum. FIG. 15—A section of cortical tissue showing the mycelium in the intercellular spaces. FIG. 16—A sugar beet seedling showing blackroot symptoms. Inoculum was introduced above the seal of paraffin and vaseline.





is kept saturated. In January 1948, inoculum was placed in artificial wounds made in beets about three months old. A plug about  $\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2}$  in. was removed, inoculum inserted, and the plug replaced. In all 16 beets so treated no rotting of the roots occurred and the plants remained free from disease as did also the checks. The inoculum that was used in this experiment was originally obtained from a seedling that had suffered from the acute phase of attack and thus this strain was capable only of producing seedling infection.

### Humidity Studies

Since infected seedlings have frequently been observed to recover and the fungus is intercellular in the host tissue, it seemed likely that humidity might be a limiting factor in the parasitic activity of *A. cochlioides*. An attempt was made to evaluate this possibility.

An apparatus was set up to test the lowest humidity at which this fungus would grow. Saturated air was passed through appropriate solutions in a series of Pettenkoffer tubes, from each of which the air emerged at a definite humidity (9). The air was blown over the fungus, which was growing on a thin piece of agar on the side of a test tube. By this method it was found that the growth of the fungus ceased when the relative humidity dropped to 95% or below. For more precise measurements another apparatus was used.

Further studies were carried on in Petri dishes in an incubator at 25° C. About 50 cc. of sulphuric acid of desired concentration (16) was poured in the tops of Petri dishes. Other inverted Petri tops, containing a thin layer of potato dextrose agar, were placed exactly over the acid dishes and the two tops were then sealed with vaseline. After two weeks, the plates were inoculated with *A. cochlioides* on a small piece of agar. Growth continued for a very limited time at all humidities, but soon stopped at a humidity of 98.7% whereas at 99.1% the fungus grew across the plate in all cases.

To check the above results, infected seedlings were placed in 0.0, 0.1, 0.2, . . . . . 1.0 *M* solutions of sucrose at 25° C. Development of the fungus gradually diminished from the 0.2 to 0.6 *M* solution in which there was no growth. When the infected seedlings were taken from the 0.6 molar solution and put in sterile water, no growth occurred, the fungus apparently having been killed. A 0.6 *M* sucrose solution has an osmotic pressure of 15.6 atmospheres and an equivalent relative humidity of 99.0% (15). These results therefore correspond fairly closely with those obtained with the acid solutions. Since the humidity of the intercellular spaces of plants may be below 99% (17), humidity may frequently limit the development of *A. cochlioides* when the soil and atmosphere become dry.

### Temperature-Growth Relations

Since field evidence indicated that temperature was frequently a limiting factor in the development of blackroot, the temperature relations of *A. cochlioides* and of disease incidence were investigated. The fungus was grown at temperatures of 5°, 10°, 13°, 16°, 20°, 22.5°, 25°, 27°, 29°, 31°, 33°, 35°, 37°,

40°, 43° C. on a thick layer of potato dextrose agar in order that the air and media would remain moist throughout the experiment. Fig. 17 shows the average diameter of the colonies after 48 hr. A parallel series on cornmeal

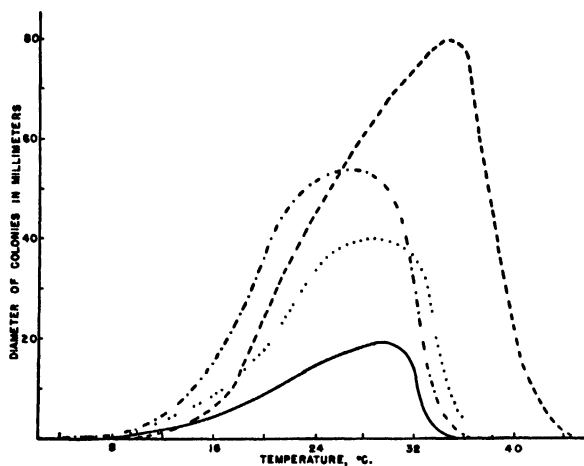


FIG. 17. Influence of temperature on the rate of growth of the four pathogens on potato dextrose agar; *A. cochlioides* —————; *R. solani* .....; *P. ultimum* -----; *P. aphanidermatum* - - - - -.

extract agar gave similar results. However, it is believed that the vegetative development of *A. cochlioides* may be only a relatively unimportant phase in its life history and that the mycelium very infrequently grows through the soil since vegetative hyphae are never sent out from diseased seedlings when they are placed in water and since this organism is only pathogenic in wet or saturated soil. It was apparent that the abundance and rate of production of zoospores should also be compared at the various temperatures. Infected seedlings were placed in sterilized water in Petri plates at the above temperatures and the times required for primary zoospore encystment, for the release of the zoospores from the cyst, and the germination of the zoospores were recorded. All three of these measurements followed the same general curve. The rate of primary zoospore encystment that could be measured most accurately is shown in Fig. 18.

The optimum temperature for mycelial growth was at least 6° C. higher than the optimum temperature for spore formation. It was observed also that the number of sporangia and zoospores formed diminished markedly on either side of this optimum temperature. This curve furthermore cut away very sharply at its limits. Since both the number of spores and the rate of their production are very important in disease spread, and since mycelial growth must occur within the host, both should be correlated with pathogenicity.

In Wisconsin temperature tank experiments naturally-infested blackroot Brookston clay loam soil was used and temperatures were maintained at

5°, 9°, 13°, 17°, 21°, and 25° C. In each tank two canisters of soil were kept dry, three at optimum moisture content, and three were kept moist, the moisture content being 20, 30, and 43% dry weight, respectively. Sixty

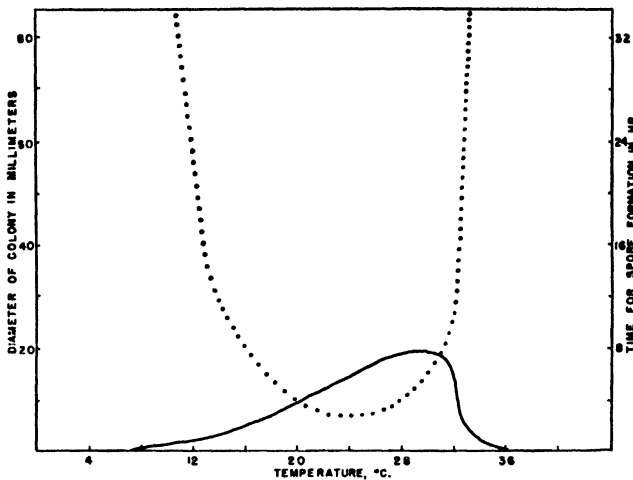


FIG. 18. Influence of temperature on the time required for *A. cochlioides* to produce groups of encysted zoospores following the immersion of infected seedlings in water . . . . . ; the influence of temperature on mycelial development —————.

sugar beet seeds were planted in each canister and the experiment was repeated. Seeds were soaked in water a day previous to planting in order that emergence might be uniform in the dry soil.

As Table II shows, no blackroot occurred in the dry soil but as the moisture content was raised, the disease became more severe. At 13° C. and below there was only a slight amount of injury but at 17° C. blackroot was becoming

TABLE II

THE PERCENTAGE BLACKROOT IN NATURALLY-INFESTED BROOKSTON CLAY LOAM SOIL AT VARIOUS TEMPERATURES AND MOISTURES

Soil moisture	Temperature, ° C.					
	5	9	13	17	21	25
Dry - 20% water content	0	0	0	0	0	0
Optimum - 30% water content	0	1	0	6.4	8.1	11.5
Moist - 43% water content	0	1.3	4.4	18.8	32.5	30.1

serious and at 21° and 25° C. it was very severe. Judging from these results the rate of spore production appears to be most directly related to disease incidence.

### *Epiphytotics*

In Ontario, blackroot usually occurs in late spring epiphytotics. At this time serious outbreaks can be expected a few days after a rain if in the meantime the weather remains warm. From this it was apparent that a high soil temperature and abundant moisture are necessary for disease development. Fig. 19 gives the daily rainfall, soil temperature, and disease incidence during

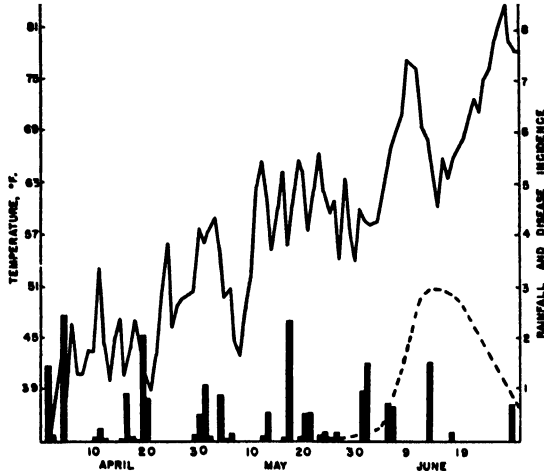


FIG. 19. The influence of soil temperature and rainfall on disease incidence, one inch of rainfall being equal to 2; ----- disease incidence, approximate acreage initially attacked at the times indicated, the ordinate 1 = 600 ac.; ————— temperature.

April, May, and June in the spring of 1947. Rainfall was frequent and abundant except for a short time at the end of June, when moisture became a limiting factor. Beet seed was planted more or less throughout the whole period and thus there were seedlings in the susceptible stage at all times. Inoculum was present in a great many of the fields, so that temperature apparently was the limiting factor until about June 8, when a serious disease outbreak began. Previous to this date the soil temperature had exceeded 60° F. only a few times and for very short periods. Below 60° F. *Aphanomyces*, as Fig. 18 shows, develops very slowly, whereas at higher temperatures the growth rate increases very rapidly. From the 7th to the 14th of June, conditions were ideal for a disease outbreak inasmuch as the soil temperature was high, moisture abundant, and many of the seedlings were in their susceptible period. Later in June the outbreak decreased because the soil was drier and the seedlings were passing into a resistant stage. In all the blackroot epiphytotics that have been under observation the same interplay of soil moisture, temperature, and host disposition has been apparent.

### *Host Range of A. cochliformis*

Some of the wild and cultivated plants that occur commonly in the sugar beet district were tested for susceptibility in sterile soil inoculated with

*A. cochlioides*. Sugar beet seed was planted along with the other seeds to ensure that *A. cochlioides* was present. Only lamb's quarters (*Chenopodium album* F.) and pigweed (*Amaranthus retroflexus* L.) were susceptible. *Chenopodium album* is in the same family as *Beta vulgaris* and becomes resistant at about the same stage in its development, but *Amaranthus retroflexus* is in the *Amaranthaceae* and is susceptible only during the first two weeks of development. Alsike, alfalfa, white Dutch clover, yellow and white sweet clover, red clover, sweet pepper, soybean, garden pea, wild carrot, dandelion, barley, fall and spring wheat, oats, yellow foxtail, timothy, and sweet corn were found to be immune. Since blackroot is only destructive in fields that have continually or frequently been planted with sugar beets, it appears that the weed hosts are not very effective in the build-up of this organism.

### Seedling Blight Caused by *Pythium aphanidermatum*

#### *Symptoms*

*Pythium aphanidermatum* attacks the seedling during its primary development, and may cause pre-emergence or postemergence blight. The embryo may be invaded before it has had time to germinate, and frequently the seedling is attacked before the cotyledons push out of the soil. The fungus may even attack the cotyledonary leaves, but more frequently it is the young root that suffers from primary infection. In a few hours after invasion the whole seedling has a water-soaked appearance and is killed.

Postemergence blight also proceeds very rapidly and 24 hr. after the first indication of wilting the seedling is almost completely decayed. Most of the seedlings are infected a day or two after emergence. Infection begins in the root, and as the moist decay advances up the hypocotyl, the seedling wilts, in a short time falls over, dries, and shrivels so completely that it is soon difficult to find any remnant of it.

If the seedlings become infected a few days later or when they have begun to produce their first pair of true leaves, they display different symptoms. In most seedlings the disease progresses so rapidly that the plant collapses at the ground level and falls over before the seedling has begun to wilt. In some cases, however, the seedling may be held upright by the surrounding soil, the cotyledonary leaves meanwhile becoming bluish in color, and a day or two later the desiccated plant falls over. In all these plants the roots are completely destroyed. The disease symptoms are shown in Fig. 20.

#### *Life History of Pythium aphanidermatum*

It was found that *P. aphanidermatum* could be readily isolated from infected tissues by the method already described for the isolation of *A. cochlioides*. It can be isolated also on agar media, but this involves more materials and labor than the water culture method, and several days are required to identify the pathogen, whereas in water it can be recognized within a day.

If a seedling, infected with *P. aphanidermatum*, is placed in water, on its surface abundant branched nonseptate mycelium begins to form at once, and within a few hours irregular lobulate inflated sporangia of varied size are present (Figs. 21 and 22). These sporangia mature very rapidly; protoplasm can be observed flowing in the mycelial threads and is shunted into the many enlarging sporangia. On each sporangium an evacuation tube forms (Fig. 23), and at its tip a vesicle arises (Fig. 24). The protoplasm from the sporangium flows into the vesicle and within a minute its development may be completed. The protoplasm in the vesicle is at first very dense and uniform in appearance and is in continuous motion. After a couple of minutes the zoospores are delimited and about four minutes later movement of the individual zoospores begins and soon a vigorous lashing of flagella can readily be detected. The activity of the spores increases extremely rapidly, they jostle violently within the vesicle, and in a minute or two the vesicular membrane breaks; the zoospores escape in rapid succession (Fig. 25) and swim swiftly in all directions. They are about  $12\ \mu$  long and  $7.5\ \mu$  wide and have two lateral flagella. After swimming for a few hours, the zoospores encyst and then germinate in the manner shown in Fig. 26.

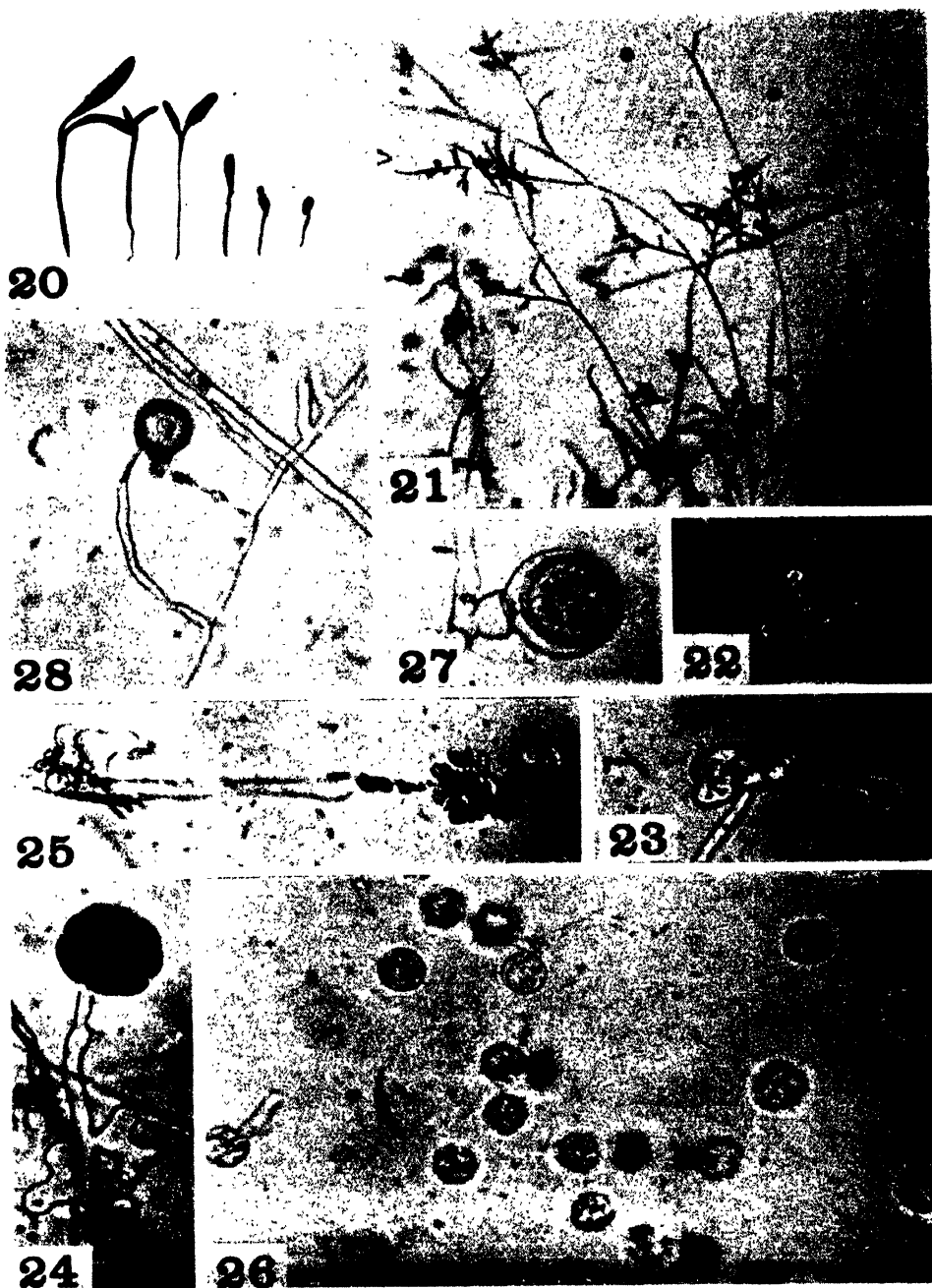
It was observed that immediate germination of the sporangia took place only when the infected seedling was placed in a small amount of water in the Petri dish so that the mycelium and asexual reproductive organs were floating at or near the surface of the water. If the diseased tissue is immersed in water so deeply that the mycelium and the sporangia are completely submerged, germination is greatly retarded and frequently suppressed.

The above description of asexual reproduction is not applicable to spore discharge at low temperatures. At  $10^{\circ}\text{C}$ . or slightly below, the zoospores after delimitation do not begin to move but remain quiescent within the vesicle for several minutes. The vesicle then breaks, the motionless zoospores merely drift away, and do not germinate as long as this low temperature is maintained. At  $12^{\circ}\text{C}$ . the normal method of zoospore discharge is carried on, and the spores are motile. Thus it appears that the breaking of the vesicle is not the result of the bombardment with zoospore-, because here the vesicle bursts even though the zoospores remain motionless.

After one or two days asexual reproduction stops and sexual organs begin to appear abundantly on the external mycelium, but they usually do not develop within the host as was so common in the case of *Aphanomyces*. A

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FIG. 20. Seedlings killed by *P. aphanidermatum* blight. The seedling on the right suffered from pre-emergence blight and even the cotyledonary leaves were infected. The oldest seedling on the left suffered only from root infection. FIG. 21. Mycelium, sporangia, and vesicles of *P. aphanidermatum* produced soon after an infected seedling is immersed in water. FIG. 22. A typical sporangium. FIG. 23. A sporangium with an evacuation tube and at its tip a vesicle beginning to form. FIG. 24. A newly-formed vesicle in which the zoospores are just beginning to be delineated. The empty sporangium and evacuation tube is also shown. FIG. 25. Zoospores breaking out of a vesicle. FIG. 26. A group of encysted zoospores and some that have germinated. FIG. 27. An oogonium and an intercalary antheridium with its penetration tube. FIG. 28. An apical oogonium and antheridium.







typical intercalary barrel-shaped antheridium with a conspicuous penetration tube is shown in Fig. 27. They measure  $9-11 \times 10-14 \mu$ , are usually monoclinal but sometimes are declinal and one or two are present with each oogonium. The oogonia, which are generally terminal (Fig. 28), but occasionally intercalary, are 22 to 27  $\mu$  in diameter. The oospores are aplerotic, 17 to 19  $\mu$  in diameter, and germinate by a germ tube.

### *Host-Parasite Relations*

Colonization of a seedling by *Pythium* is achieved through the following sequence of events. A zoospore comes to rest on the surface of a root, produces a germ tube, and soon an appressorium forms on the epidermis. A penetration tube develops at the base of the appressorium and pushes through the epidermal wall where it enlarges and continues to grow intracellularly through the cortex and even into the stele. The mycelium is constricted where it passes through the cell walls and is to be found in advance of the necrotic symptoms.

Seedlings grown at a low temperature are susceptible for a considerably longer time than those produced at high temperatures but in any case they become resistant when the first pair of true leaves are about  $\frac{1}{4}$  in. long, at which time, according to Artschwager (1) primary growth of the beet is concluded. This resistance might be of a physiological or a mechanical nature. If it were mechanical it would appear to be associated with the development of a suberin lamella over the entire surface of each endodermal cell, with the exception of the root tip, which retains a primary endodermis. To enquire into this possibility, seedlings were grown in sterile soil that was saturated with water so that oxygen would be excluded from the roots. It was hoped that, in the absence of oxygen, fatty acids could not be changed into suberin, in which case the roots might still be susceptible. After the seedlings had passed their usual susceptible stage, *P. aphanidermatum* inoculum was put in the soil but no blight occurred. However, it was found that suberin did form in the roots that were growing in water. Even if the fungus could not penetrate the suberin lamella, entrance should still be possible through the root tips that are unsuberized, through transfusion cells in the older epidermis, or through wounds produced by the emergence of secondary roots. This suggests that resistance must be due to some physiological condition that arises at the beginning of secondary growth.

### *Epidemiology*

Both high soil temperature and abundant moisture are necessary for an epiphytotic of blight in the Exeter sandy loam soil. In spite of this, and although the seedlings are susceptible to *P. aphanidermatum* for only a very short time, nevertheless whole fields of sugar beet seedlings may be destroyed.

The necessity of abundant moisture is apparent since only under such conditions could zoospore production or liberation occur. The growth-temperature curve for mycelial development (Fig. 29) shows further that

high temperature likewise favors rapid growth of *P. aphanidermatum*, whose optimum temperature of 34° to 35° C. is unusually high and at least 6° to 8° C. higher than the optimum of the other sugar beet pathogens studied. How-

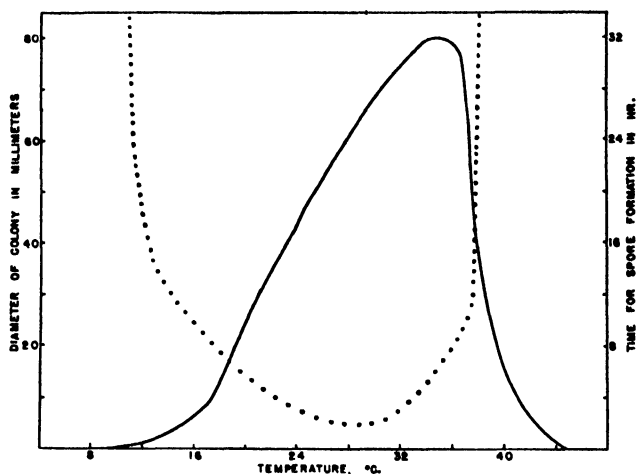


FIG. 29. Influence of temperature on the length of interval required for *P. aphanidermatum* to produce zoospores from the time infected seedlings are immersed in water . . . . . ; the influence of temperature on mycelial development ———.

ever, *P. aphanidermatum* develops very rapidly over a wide range of temperatures with its lower limit only slightly higher than that of the other pathogens (Fig. 17).

Since the zoospores play such an important part in the spread and build-up of this pathogen, the time required for zoospores to form at various temperatures after naturally-infected seedlings were placed in water was determined. The curve representing the rate of zoospore production is shown in Fig. 29 and its optimum is much wider and about 6° C. lower than that for mycelial development. At temperatures on either side of the optimum, the time required for zoospore production increased and also the number of sporangia and zoospores formed diminished extremely rapidly. For example, at 16° C. there was only about one-fifth as many sporangia formed as at 29° C.

Pathogenicity of this organism was tested in naturally-infested sandy loam soil in Wisconsin temperature tanks. The soil was kept very moist throughout the experiment and air temperature was maintained at about 27° C. Sixty seeds were planted in each of eight canisters of all tanks.

The average results of two experiments are given in Table III from which it is apparent that the greatest amount of blight occurred at 25° and 30° C. with only a slight amount at 15° and 37° C. At 10° C. no blight occurred, which was to be expected since at this temperature the fungus grows very slowly.

TABLE III

EFFECT OF TEMPERATURE ON THE INCIDENCE OF SEEDLING BLIGHT IN MOIST SANDY LOAM SOIL NATURALLY INFESTED WITH *P. aphanidermatum*

Temperature, ° C.	Postemergence blight, %	Final stand, %
37	6.9	90.8*
30	36.2	48.7
25	33.9	45.1
20	27.0	61.3
15	4.1	94.1
10	0.0	98.2

\* Germinating capacity of seed 98%.

In these experiments blight reached a maximum within a day or two after the day on which the largest number of seedlings emerged. This correlation between emergence and blight has been consistently encountered in flat experiments.

### Diseases Due to Other Pathogens

#### *Pythium ultimum*

Disease symptoms resulting from *P. ultimum* attack resemble those on seedlings infected with *P. aphanidermatum* and again the seedlings become resistant when secondary growth begins. *P. ultimum* is a low temperature fungus, as Fig. 17 shows, and it may cause blight even at 5° C.

The pathogen is readily identified in water and it produces many sporangia and oogonia on the mycelium that passes out from the host (Fig. 30). The sporangia are usually terminal, about 20  $\mu$  in diameter and always germinate by a germ tube. The oogonia (Fig. 31) are spherical, generally terminal, and approximately 21  $\mu$  in diameter and contain a single aplerotic oospore that has a single central reserve globule and a refringent body. There is usually one antheridium per oogonium; it is curved, monoclinous, and arises immediately beneath the oogonium.

*P. ultimum* was found in all the clay soils studied and even in the marl soil of the Blackwell area but in spite of this wide distribution it is of little economic importance. This organism may cause a considerable amount of blight in the greenhouse but during the past three seasons it has not caused serious damage in any sugar beet field. In the marl soil, rootrot is not a problem whatsoever but under greenhouse conditions *P. ultimum* can be readily isolated from infected seedlings and this may be due to the higher soil temperature under which the beets are grown.

#### *Rhizoctonia solani*

This organism is also widely distributed and of little economic significance. However, it may infect beets at any age, the point of attack usually being at the ground level (Fig. 32). On seedlings it produces dry local lesions that

completely cut off the beet at the soil line. On lesions that may occur on large beets the mycelium can frequently be seen. This fungus grows well on infected tissue in water and can be identified by the angle the branches form to the main hypha, the constriction of the branches where they join the main thread, and the uniform placement of the first septum in the branch (Fig. 33).

Although *R. solani* isolates were extremely virulent in flat tests in which the soil had been sterilized previous to planting and despite the wide distribution of this pathogen, it caused an exceedingly small amount of rootrot. This suggests that some other soil organisms may antagonize or prohibit the development of *R. solani* especially since Weindling (19) found that *Trichoderma lignorum* antagonized the development of *R. solani* and Warren (18) frequently isolated from fragments of diseased beets a species of *Papulospora* along with *R. solani*; this species, he showed, was parasitic on *R. solani* and effective in reducing the amount of sugar beet rootrot *R. solani* caused. Low temperature may also limit the parasitic activity of *R. solani* in the field because the soil temperature during seedling development is usually below 65° F. and this fungus develops only at one-third of its optimum rate at this temperature. This is supported by the fact that Leach (11) states that in Central California this fungus is rarely recovered from early spring plantings but frequently from later ones.

### The Distribution of Pathogen in Relation to Depth and Soil Type

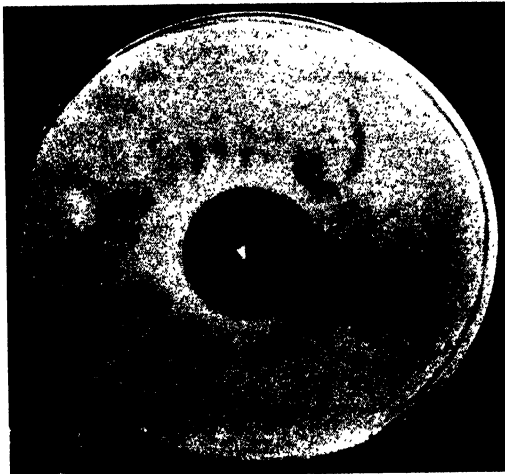
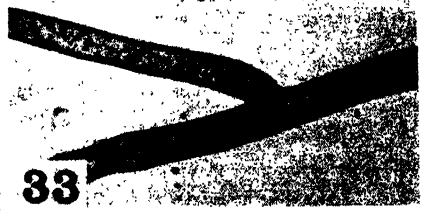
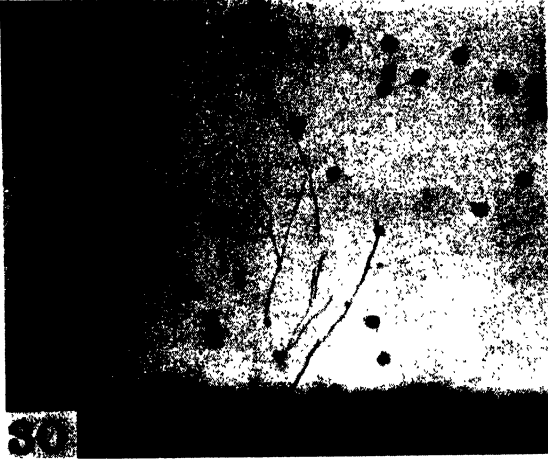
To investigate the abundance of pathogens at different depths, soil samples were taken from four profiles in two Brookston clay loam rootrot fields, and from four profiles from Exeter sandy loam. For each profile, holes were dug about 2½ ft. deep and one side was smoothly squared perpendicular to the soil's surface. Soil samples were removed by means of a cork borer at 2-in. intervals and in each profile the dividing line between the top and subsoil was noted. Inasmuch as these pathogens cannot be isolated directly from the soil, the number of seedlings that damped-off in these soil samples was used as the measure of the abundance of the pathogens. The soil samples were put in 2-in. glass caps; sugar beet seed was planted in each and in order that rootrot would be at its maximum the soil was kept very damp.

In the Exeter sandy loam the top soil was approximately 8½ in. thick and *P. aphanidermatum* produced blight equally readily in all samples taken from this layer, whereas none occurred in the subsoil. Dilution plates showed that

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FIG. 30. Asexual and sexual organs on the mycelium of *P. ultimum* soon after infected seedlings were placed in water. FIG. 31. A typical antheridium and oogonium of *P. ultimum*. FIG. 32. Seedlings that have suffered from *R. solani* attack. Infection occurred at the ground level. FIG. 33. Typical *R. solani* mycelium, note the constriction at the origin of the branch, the 45° angle that it forms with the main thread and placement of first septum, in branch. FIG. 34. Response of sugar beets in increased stand and enhanced vigor to the incorporation of soybean residue (Flats 1) as contrasted with untreated soil (Flats 2). FIG. 35. *A. cochlioides* growing around a lump of Arasan on a Petri plate. FIG. 36. The retardation in growth of *P. aphanidermatum* is shown as it approaches the Arasan.

PLATE V





bacteria were twice as numerous and fungi 50 times as abundant in the surface soil as in soil 13 in. deep. The dilution plate technique is described in the succeeding section.

In the Brookston clay loam soil *A. cochlioides* and *P. ultimum* likewise were found only in the top soil and, as shown in Table IV, were most abundant in the top two inches of surface soil.

TABLE IV

FREQUENCY OF ISOLATION OF *P. ultimum* AND *A. cochlioides* AT DIFFERENT DEPTHS IN ROOTROT BROOKSTON CLAY LOAM SOIL

Depth, in.	<i>P. ultimum</i>	<i>A. cochlioides</i>
$\frac{1}{2}$	112	72
2	97	54
4	71	25
6	26	20
8	20	0

As was indicated in Table I the distribution of sugar beet pathogens was closely correlated with soil type; thus *A. cochlioides* was isolated from clay soil only, *P. ultimum* from the clay and marl soils, and *P. aphanidermatum* from the Exeter sandy loam in which it was the only pathogen present.

Although *P. ultimum* and *A. cochlioides* are not found in the sandy loam soil, under greenhouse conditions they will survive in it along with *P. aphanidermatum* for at least one year. It is difficult to understand why they are not present in this sandy loam. However, since sugar beets have only been grown in this area for a few years perhaps sufficient time has not elapsed for these two pathogens to increase in abundance or to have spread into this area. This explanation does not seem too satisfactory inasmuch as *P. aphanidermatum* is already very abundant. However, the build-up of *P. aphanidermatum* may have occurred on some other host that is commonly grown in this locality, or possibly *P. aphanidermatum*, which is one of the high temperature *Pythium* spp. (13), is better adapted than the other pathogens to the high temperatures that prevail in this light sandy soil.

To test the survival of *P. aphanidermatum* in clay soils a cornmeal sand culture was mixed with some University of Toronto clay in October 1947. Abundant blight of sugar beets, which were planted in it immediately after inoculation, occurred. The soil was then divided in five portions; two were put in a room at about 25° C. and one portion was kept moist while the other one was allowed to dry; two were treated likewise in a 5° C. room; and one portion was put outside. An equal quantity of Brookston clay loam soil received the same treatment.

In April 1948 the presence of *P. aphanidermatum* in these soils was tested by planting sugar beet seed in them. The soil was kept moist in order to increase the amount of blight, and the Brookston clay loam soil was put in



canisters in a Wisconsin temperature tank maintained at 35° C., so that no rootrot would be produced by the lower temperature pathogens. Since blight did not occur in either soil, *P. aphanidermatum* evidently did not survive six months in these clay soils. Since the persistence of specific fungi in a given soil environment seems to be the resultant of a large number of physical, chemical, and biotic factors, it would require substantial and far-reaching investigations to determine in this case what is basic to the correlations between soil types and pathogen distribution. It is felt, however, that the evidence presented strongly indicates that the correlations are authentic and the distributions hence are likely to remain functions of the soil types.

### Soil Microbiological Studies

The soil in the vicinity of the roots usually supports noticeably higher numbers of microorganisms than soil more distant from the plant. The zone close to the roots and under the influence of their excretions has been called the rhizosphere. With strawberry rootrot it has been found that the flora in the rhizosphere of diseased roots is greater than that of healthy roots (8). It appeared that a study of the rhizosphere of beet roots in relation to infection might be of value.

Using the standard technique, rhizosphere studies were made of healthy roots and ones just beginning to show disease symptoms, on the 8th, 11th, and 14th day after planting. The seedlings were grown in Brookston clay loam and were watered moderately. Dilutions of 1 : 10,000 were plated for fungus counts in Petri dishes of peptone-glucose agar (5), and Czapek's agar modified by reducing the sucrose content to 1%. The peptone-glucose Czapek's agars were sterilized in Erlenmeyer flasks that contained 100 cc. of media and, preparatory to pouring in Petri dishes, the pH was adjusted to approximately 4 by the addition of  $\frac{1}{2}$  cc. of normal sulphuric acid to each. Dilutions of 1 : 1,000,000 were plated in Petri dishes of sodium albuminate (5) for counts of bacteria and actinomycetes.

The results are summarized in Table V, which shows that bacteria were much more numerous in the rhizosphere of the diseased than the healthy roots and the difference became more marked as the plants became older. The number of fungi in the rhizosphere of diseased seedlings was significantly lower than in that of the healthy ones on the eighth day and no appreciable variation in number occurred later. Furthermore, in the rhizosphere of the healthy seedlings the number of fungi declined as the seedlings grew older until on the 14th day they were less abundant than in the case of the diseased seedlings. Inasmuch as the symptoms at that stage are indistinguishable, the diseased seedlings might have been infected with either *A. cochlioides* or *P. ultimum* or both and each of these might have exerted a specific but markedly different influence on the rhizosphere. It is believed, however, that *P. ultimum* caused most of the infection since the soil was watered moderately and the seedlings were young.

TABLE V

COMPARISON OF THE RHIZOSPHERE POPULATION OF DISEASED AND HEALTHY SUGAR BEET SEEDLINGS AT DIFFERENT AGES GROWN IN BROOKSTON CLAY LOAM

Age of seedling in days	Diseased seedlings			Healthy seedlings		
	Fungi on:		Bacteria and actinomycetes	Fungi on:		Bacteria and actinomycetes
	Peptone-glucose agar	Czapek's agar		Peptone-glucose agar	Czapek's agar	
8	22*	20	137**	37	33	99
11	27	19	159	19	14	87
14	17	21	283	13	14	115

\* Number of fungi in  $1/10^4$  gm. of soil; \*\* number of bacteria in  $1/10^4$  gm. of soil; each is the average count in five Petri plates.

The cause of the increased numbers of bacteria in the rhizosphere of seedlings immediately after the initiation of infection is by no means obvious. It could result from some common influence exerted by the pathogen; it might reflect merely local increases in nutrients associated with the necrosis of the roots, or it might be due to quite unrelated factors or to a combination of several of them. It seemed, however, that a study of the rhizosphere of roots that were killed artificially might aid in evaluating the influence of the necrotic factor in comparison with that of the parasite.

Sugar beet seed was planted close to the glass plates in miniature root-observation boxes (12), which were about 3 in. high, 1 in. thick, and 3 in. long. The glass plates that formed the two sides of these boxes fitted in wooden grooves on either side and could readily be pulled upwards, thus exposing the roots on the side, in the region of the seed. At about the soil line, the hypocotyl of the seedling was frozen for a few seconds over a distance of one-quarter of an inch. Liquid carbon dioxide was used for this purpose, because it left no chemical residue and had no affect on the soil flora. The freezing was done by touching the hypocotyl with the convex side of a pipette whose tip curved through  $180^\circ$  and through which carbon dioxide passed causing ice to form on its surface. Since this ice did the freezing the gas stream did not touch the seedling or the soil. This treatment was carried on between the 10th and 25th day after planting in Exeter sandy loam soil in which only the one pathogen *P. aphanidermatum* was present.

Rhizosphere studies were made one, two, three, and four days after treatment and in one series parallel rhizosphere counts were made of healthy seedlings (Table VI). In the first series both fungi and bacteria increased in the rhizosphere of the frozen seedling from the time of treatment. With the fungi this was not very marked, but the increase in the number of bacteria and actinomycetes each day was enormous and reached 700% within three days. In the second series, rhizosphere studies were again made of both healthy and frozen seedlings after periods of one, two, three, and four days. The first

TABLE VI

COMPARISON OF THE RHIZOSPHERE POPULATION OF SUGAR BEET SEEDLINGS GROWN IN EXETER SANDY LOAM, ONE, TWO, THREE, AND FOUR DAYS AFTER LOCAL FREEZING

Days after treatment	Fungi on:				Bacteria and actinomycetes	
	Peptone-glucose agar		Czapek's agar		Frozen	Healthy
	Frozen	Healthy	Frozen	Healthy		
1	18*	—	13	—	37**	—
2	23	—	16	—	178	—
3	24	—	24	—	246	—
1	74	86	70	68	93	91
2	36	52	37	48	135	65
3	68	26	52	19	190	36
4	80	27	75	15	295	41

\* Number of fungi in  $1/10^4$  gm. of soil;    \* number of bacteria in  $1/10^6$  gm. of soil; each is the average count on five plates.

day after treatment the number of organisms was about equal in the rhizospheres of the frozen and healthy seedlings, but, as time went on, a greater difference in numbers became apparent, especially in the case of the bacteria, but there was a gradual decrease in numbers in the rhizosphere of the healthy seedlings, the reason for which is not immediately apparent.

Table VII gives comparative figures in individual experiments of diseased, frozen, and healthy seedlings taken two and three days after treatment. In the rhizosphere of the frozen seedlings the numbers of bacteria and actinomycetes were much more numerous on the third than on the second day; thus they were 430% greater than those of the healthy seedlings on the third day, whereas they had been only 240% greater on the second day. The diseased seedlings supported a flora that was much larger than that of the healthy seedlings and slightly larger than that of the frozen ones, although the difference was not nearly so pronounced in the third day after freezing.

The results show that the death of the seedlings, accomplished artificially, induced closely approximate quantitative effects in the rhizosphere as did death caused by the pathogen. In both cases the numbers of organisms increase from day to day after necrosis has started and this increment is very great with bacteria and actinomycetes, but is only slight with fungi. Thus it is apparent that the utilization of necrotic tissue and substances released in this breakdown probably contributes substantially to the marked increase in the number of microorganisms in the rhizosphere of diseased seedlings. The death of beet seedlings in sandy loam soil caused by *P. aphanidermatum* produced a quantitative rhizosphere response comparable to that produced by seedlings killed in Brookston clay loam by *P. ultimum* and *A. cochlidioides* and this would be expected if the necrotic tissue, which in both cases is produced so soon after infection, is responsible for this rhizosphere effect.

TABLE VII

A COMPARISON IN THE NUMBER OF FUNGI, BACTERIA, AND ACTINOMYCETES IN THE RHIZOSPHERE OF HEALTHY, DISEASED, AND FROZEN SEEDLINGS GROWN IN EXETER SANDY LOAM, AT TWO AND THREE DAYS AFTER FREEZING

Days after treatment	Fungi on:						Bacteria and actinomycetes		
	Peptone-glucose agar			Czapek's agar			Diseased	Frozen	Healthy
	Diseased	Frozen	Healthy	Diseased	Frozen	Healthy			
2	41*	71	15	23	19	17	333**	159	92
2	11	15	13	12	14	12	90	91	40
2	30	15	12	50	18	12	371	148	34
Av.	27	34	13	28	17	14	265	133	55
3	15	19	11	16	23	14	86	217	37
3	61	49	30	75	55	23	746	458	63
3	76	33	44	76	69	48	528	370	136
Av.	51	34	28	56	49	28	453	348	79

\* Number of fungi in  $1/10^4$  gm. of soil; \*\* number of bacteria and actinomycetes in  $1/10^6$  gm. of soil; average count of five plates in each.

### Control of Sugar Beet Rootrot

Since *Phoma betae* is not present on sugar beet seed that is used commercially in southern Ontario and, since nontreated seed, planted in steam sterilized soil, gives healthy stands of seedlings, rootrot control in this district resolves itself into one of controlling soil-borne pathogens.

It has been demonstrated that the turning in of green cover crops is very effective in changing the microbiological balance of soils. Hildebrand and West (8) under their experimental conditions reported that soybeans changed the flora to such an extent that strawberry rootrot was almost prevented and at the same time *Thielaviopsis basicola*, which is a parasite on tobacco, became much more abundant. Green rye has been reported likewise to be quite effective in a similar way in reducing the amount of scab that occurs on potatoes (14).

In an experiment two successive crops of soybeans were grown in 'rootrot' Brookston clay loam soil, and on Aug. 6 and Nov. 19, 1946, the soybeans at their maximum succulence were ground finely and incorporated with the soil in which they had grown. A similar amount of Brookston clay loam soil was untreated and both lots were kept in the greenhouse throughout the course of the experiment. Sugar beet seed was planted in each on May 26, 1947.

In the soybean treated soil the final stand was increased from 18 to 49% and, in addition, the plants were much larger (Fig. 34). It would appear that here too the soybean residue had reduced disease incidence through the shift in the microbiological balance that it must have occasioned in the experimental

soil. It is equally apparent, however, from the increased vigor of the seedlings, that other factors are operating as well and these may not be without significance in relation to the reaction of the host to the parasites.

The efficacy of two antibiotics, penicillin and streptomycin, applied as seed treatments, was tested. Preliminary tests showed that penicillin thus applied was ineffective and further investigation was carried on only with streptomycin. A stock solution of streptomycin was made by adding 10 cc. of water at a pH of 3 to 1 gm. (80,000 units) of crystalline streptomycin. Fifty seeds were soaked in each of various concentrations of this solution for 48 hr. at 5° C. previous to planting in naturally-infested Brookston clay loam soil.

The results summarized in Table VIII show that streptomycin definitely does control rootrot and that there is a wide latitude between effective and phytotoxic concentrations. A higher percentage of emergence might have

TABLE VIII

EFFECT OF SEED TREATMENT WITH STREPTOMYCIN ON INCIDENCE OF ROOTROT IN SUGAR BEETS GROWN IN NATURALLY-INFESTED BROOKSTON CLAY LOAM SOIL

Treatment	Germination, %	Rootrot, %
Stock solution	66	0.0*
" " $\frac{1}{2}$ strength	64	0.0
" " $\frac{1}{50}$ strength	72	22.2
Untreated	54	74.0

\* 6% of the seedlings showed toxic effects.

resulted if the seed had been treated at different temperatures or at a higher pH. Further tests should be conducted with both purified and impure streptomycin since, if the antibiotic were effective in its crude state, it might be economically feasible to use it commercially for seed treatments. This experiment demonstrates, however, that an antibiotic that is produced by the soil organism, *Streptomyces griseus*, can be used effectively as a seed treatment that prevents the parasitic activity of soil pathogens. This suggests the desirability of extensive studies of the potential significance of antibiotic substances as fungicides. Such antibiotic substances may well be produced by fungi and bacteria in large numbers and in a great variety of chemical patterns, and it is quite possible that they might be used effectively and economically as protective or eradicant fungicides. The possibility is all the more worthy of attention since the results have, in general, been so disappointing in the attempts to control soil-borne parasites through artificially induced shifts in the microbiological balance of infested soils.

Throughout this study it was repeatedly observed that commercial fertilizers may aid in reducing the amount of rootrot. In experiments designed to investigate their effect, however, fertilizer had no influence on pre-emergence

blight but did reduce postemergence blight considerably (Table IX). The beet was found to pass through its primary development much more quickly when grown in soil to which fertilizer had been added. It is felt that, in as

TABLE IX

EFFECT OF THE ADDITION OF COMMERCIAL FERTILIZER ON EMERGENCE AND INCIDENCE OF ROOTROT IN SUGAR BEETS GROWN IN BROOKSTON CLAY LOAM

Treatment	Emergence, %	Rootrot, %	Final stand, %
Fertilizer*	82.3	17.0	68.5
Check	84.6	55.8	37.6

\* 2-16-8 Fertilizer was applied at the rate of 400 lb. per acre.

much as this significantly reduces the time the seedlings are susceptible to *Pythium*, this is the most probable explanation of the action of fertilizers, but other possibilities have not been explored.

Hildebrand *et al.* (7) found that Arasan was very effective in controlling rootrot of sugar beets and this paper presents some preliminary experiments carried on to investigate the mechanism of action of Arasan in preventing root deterioration. Its direct action was tested by placing a small lump of Arasan in the center of a number of Petri dishes that contained potato or cornmeal extract agar. In each dish one of the pathogens was inoculated at the side and allowed to grow toward the Arasan. Growth of *A. cochlioides* was retarded initially at a distance of one inch, but after a few days it usually grew to within one-half inch of the Arasan (Fig. 35).

*P. aphanidermatum* was much retarded, but not inhibited, as it approached the Arasan (Fig. 36) and finally grew very close to it. *P. ultimum*, *R. solani*, and *Penicillium* sp. reacted in a similar way. The distance at which retardation occurred was slightly different on the two media and in the soil the reaction may be quite different. A direct fungicidal component, however, seems to account in part at least for the effectiveness of Arasan.

The method by which, in these experiments, Arasan acted in advance was not known and to test the possibility of the toxin being volatile, a thin slice of media was removed between the lump of Arasan and the pathogen. In all cases, however, this space eliminated all retardation by Arasan and thus the toxin must have been diffusing through the media.

The striking effectiveness of soil applications suggested the possibility that Arasan might be acting in part through some disturbance of the microbiological balance in the soil unfavorable to the pathogens. Accordingly, some experiments were run to find out what influence this compound had on the soil flora. Arasan at the rate of 0.07 gm. per 27.5 cu. in. air-dried soil was uniformly mixed with Brookston clay loam. This is equivalent to an application of 4 lb. per acre (7), the rate at which it is now used commercially. Then

125 cc. of water was added to this and to the untreated soil used in the check and both were kept in open flasks. At weekly intervals dilution plates were poured as described previously and counts of bacteria and fungi were taken and the experiment was replicated.

These results, which are given in Table X, show that the number of fungi in the Arasan treated soil was reduced within two days to about one-fourth of that in the check soil and that this relationship persisted even after 67 days.

TABLE X

NUMBER OF FUNGI AND BACTERIA AFTER DIFFERENT INTERVALS IN ARASAN-TREATED AND NONTREATED BROOKSTON CLAY LOAM

Days after treatment	Fungi on:		Bacteria and actinomycetes	
	Treated	Untreated	Treated	Untreated
2	1 0*	11 5	58**	42
8	2 5	5 5	80	39
15	1 5	5 5	76	19
21	0 5	4 5	55	15
29	2 0	6 0	36	21
36	2 0	10 0	31	17
67	1 0	6 5	18	17

\* Number of fungi in  $1/10^4$  gm. of soil.

\*\* Number of bacteria and actinomycetes in  $1/10^6$  gm of soil, each is the average count on eight plates.

In the case of the bacteria, on the contrary, the Arasan treatment stimulated development initially, the number reaching a maximum after about 15 days. By the 67th day the numbers had returned to normal. These results are highly suggestive and indicate the desirability of carrying this phase of the investigation further.

### Discussion and Summary

Of the four rootrot pathogens of sugar beets that were found in southern Ontario, *A. cochlioides*, which causes what is commonly called blackroot of sugar beets, is easily the most important economically. That it was not previously reported in Canada probably is due to the difficulty of isolating it by ordinary laboratory techniques. However, by the single expedient of immersing in water bits of infected tissues, not only is the fungus induced to emerge, but so also are the other three pathogens that were encountered. Even *R. solani* grows extremely well under these conditions as, unfortunately, do so many saprophytes, such as species of *Actinomyces*, *Penicillium*, and *Fusarium*. In very young infections, however, frequently only the pathogenic fungi were observed and in all cases the development of most bacteria was greatly retarded in the water and their effects on the fungi largely neutralized. This method is not only very convenient but also saves a great deal of time, inasmuch as the four pathogens can be readily identified within a few hours after the infected tissues are placed in water.

If seedlings become infected with *A. cochlioides* before they are about two weeks old, they soon die, whereas, if they are attacked at later stages, they may live for a long time and may even recover. Seedlings recover only if the air and soil moisture becomes limiting and the temperature is relatively low. In such studies as have been made of the physiology of *A. cochlioides* the most striking feature has been its marked sensitivity to relative humidities only slightly below saturation. Growth of this fungus was inhibited by a humidity of 98.7%, determined by the sulphuric acid method. Retardation of growth was detected even in a 0.2 M sucrose solution and this is equivalent to a relative humidity of 99.8%. This organism cannot survive in a sucrose solution that has an osmotic pressure of 15.6 atmospheres. *A. cochlioides* appears to be more sensitive to humidity than many of the pathogenic bacteria (4 and 15) and the fungi tested by Hawkins (6). Considering this sensitivity, humidity may be an important limiting factor in the parasitic activity of *A. cochlioides*. The intercellular humidity of the sugar beet was not measured, but the humidity of intercellular spaces in plants is quite variable. Thut (17) has reported that the intercellular humidity in unwilted leaves in some plants may be as low as 90%.

Whether penetration of *A. cochlioides* is direct, stomatal, or associated with wounds has not been definitely determined. Kendrick (10) reported that the closely related species *Aphanomyces raphani* entered the radish only through natural wounds made by the emergence of secondary roots. *A. cochlioides* must have some other portal of entry, because it infects the beet before secondary roots are produced. The fact that infection occurs in the hypocotyl, which has a thick cuticle and numerous open stomata, suggests strongly that infection may take place through the stomata. The intercellular locus of the mycelium would tend to support this view. This fungus invades only the cortical tissue of the seedling, the hypocotyl being the vulnerable point of attack, but the fungus may grow down into the cortical tissue of the root or may invade the root cortex after this has begun to slough off.

In southern Ontario the chronic phase of attack has been conspicuously lacking and even in fields in which it was most severe, has not been found to affect more than 0.1% of the beets. This virtual absence of the chronic phase contrasts sharply with the condition reported by Coons *et al.* (3) in the northeastern sections of the United States. Here the chronic effects of *A. cochlioides* are particularly serious.

Buchholtz and Meredith (2) found a marked difference in the ability of six isolates to produce the chronic phase and two of the isolates caused no rotting whatsoever. This indicates that there are strains of *A. cochlioides* whose infection capabilities vary. Such strains would seem to be more abundant in the northeastern part of the United States than in Ontario since no strain differences were obtained throughout these investigations. On the other hand, some vector may be necessary for the initiation of the chronic phase. This is suggested at least in the results of Buchholtz and Meredith (2) who



only observed typical rootrot in an extremely low percentage of beets that were unwounded, even when they used isolates that rotted all the beets when they were wounded. This possibility is worthy of further investigation.

The *Pythium* spp. are intracellular parasites and only attack the seedling during primary development. Resistance that begins simultaneously with secondary growth may be either mechanical or physiological, but is probably the latter.

The four pathogens were found only in the top soil and *A. cochlioides* and *P. ultimum* were most abundant in the two inches of soil at the surface. The distribution of the causal organisms was closely correlated with soil type, *A. cochlioides* being limited to the clay soil and *P. aphanidermatum* to the sandy loam soil. Some evidence was obtained that suggested that the explanation was probably microbiological.

The flora in the rhizosphere of the diseased seedlings was much greater, especially in bacteria and actinomycetes, than in the rhizosphere of the healthy seedlings. A comparable quantitative increase in microorganisms resulted when the seedlings were killed artificially with liquid carbon dioxide, which suggested that the utilization of necrotic tissue probably contributes substantially to the noticeable increase in the flora.

Streptomycin, which is produced by *Streptomyces griseus*, was very effective as a seed treatment and prevented rootrot by soil pathogens. The potential significance of antibiotic substances and the possibility that they might be used effectively and economically as protective or eradicator fungicides is stressed.

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## STUDIES IN FOREST PATHOLOGY

### VII. DECAY IN WESTERN HEMLOCK AND FIR IN THE FRANKLIN RIVER AREA, BRITISH COLUMBIA<sup>1</sup>

BY D. C. BUCKLAND,<sup>2</sup> R. E. FOSTER,<sup>3</sup> AND V. J. NORDIN<sup>4</sup>

#### Abstract

An investigation of decay in western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and fir (mainly *Abies amabilis* (Loud.) Forb.) in the Juan de Fuca forest region of British Columbia has shown that the major organisms causing root and butt rots are the same in both species. These are *Poria subacida* (Peck) Sacc., *Fomes annosus* (Fr.) Cke., *Armillaria mellea* Vahl ex Fr., *Polyporus sulphureus* Bull. ex Fr., and *P. circinatus* Fr. Those organisms causing trunk rots of western hemlock, in decreasing order of importance, are *Fomes pinicola* (Sw.) Cke., *F. Pini* (Thore) Lloyd, *Stereum abietinum* Pers., *Fomes Hartigii* (Allesch.) Sacc. and Trav., and *Hydnum* sp. (*H. abietis*). These same organisms causing trunk rots of fir, in decreasing order of importance, are *Fomes pinicola*, *Stereum abietinum*, *Hydnum* sp. (*H. abietis*), *Fomes Pini*, and *Fomes Hartigii*. The logs of 963 western hemlock were analyzed in detail. Maximum periodic volume increment was reached between 225 and 275 years of age. Maximum periodic volume increment was reached between 275 and 325 years of age in the 719 fir that were analyzed. Scars were the most frequent avenue of entrance for infection. In 59% of the cases of infection studied the fungus had entered through wounds.

#### Introduction

In 1945 a request was made by logging interests in the Juan de Fuca forest region that information be obtained on the decays of western hemlock (*Tsuga heterophylla* (Raf.) Sarg.) and fir (mainly *Abies amabilis* (Loud.) Forb.). Basic knowledge of the decay loss in stands of various ages was particularly desired for working out cutting schedules in a sustained yield forestry program. To obtain the information needed for the efficient utilization of the 500,000 acres in question, it was necessary that precise inventories of the region be made. The accuracy of forest inventories depends largely on the accuracy with which the expected loss through decay can be predicted. It was the main object of this study, therefore, to determine as closely as possible the loss through decay in relation to age and diameter in western hemlock and fir. Wide variations in the cause and extent of decay in a given host are known to exist in different regions. It was not possible, therefore, to use the results of previous investigations (3, 4, 6, 11) to answer the request. The field work was carried out in 1945 and 1946 and the information obtained is presented in this paper.

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### Location of Studies

It has been demonstrated repeatedly that wide variation in the growth of a tree species and in the cause and extent of decay occurring in it may exist between regions. Therefore the field work in this study was confined to one region containing as nearly as possible a uniform forest type. The study was carried out mainly in the Franklin River area through to the Nitinat River area, in the northwestern portion of the Juan de Fuca forest region (Text-fig. 1) on southern Vancouver Island, B.C.

Thirteen areas containing 26.1 acres were analyzed. These study areas were situated in four valleys or centers as indicated in Text-fig. 1. The first, a valley running from the headwaters of the Sarita River to Coleman Creek, contained 13.0 acres in six study areas. The second, a valley running north-east of Darlington Lake, contained 7.5 acres in four study areas. The third, in the Francis Lake valley, contained 1.6 acres in one study area. The fourth, located south of the Nitinat River, contained 4.0 acres in two study areas.

### Forest Type

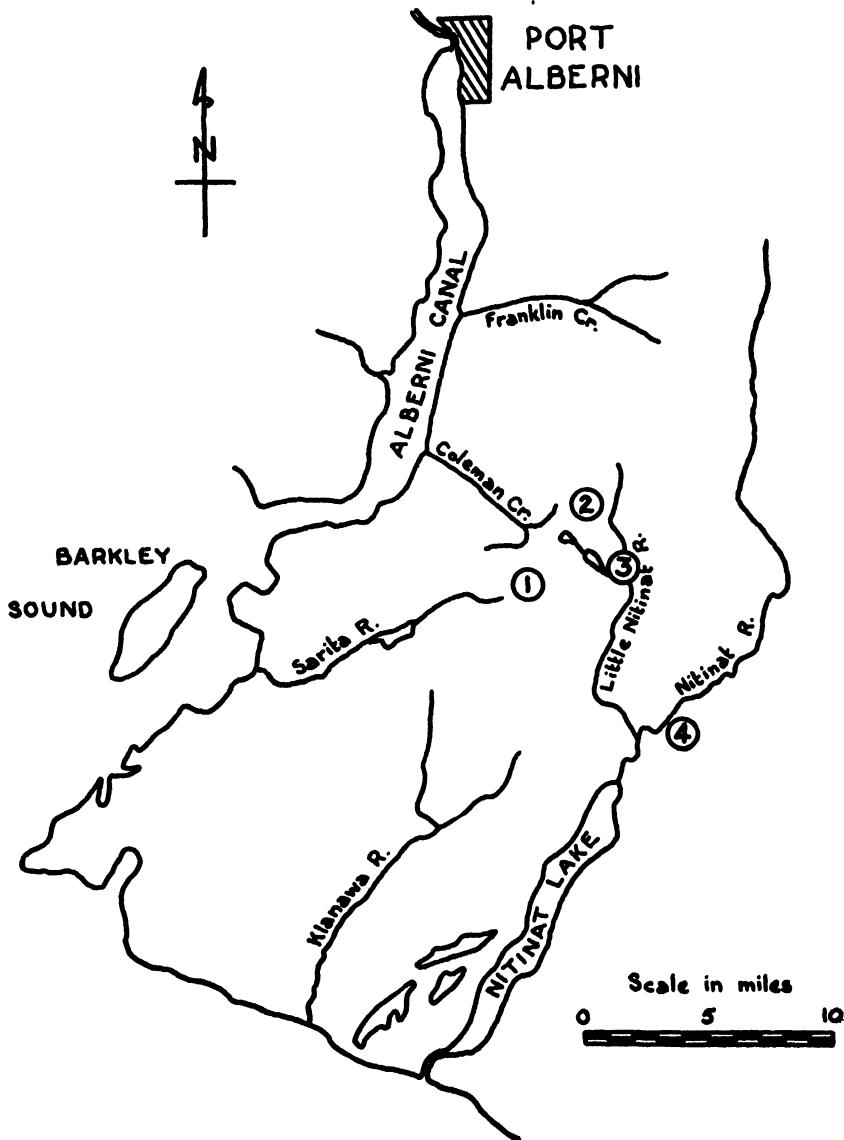
The Juan de Fuca forest region contains heavy stands of coniferous timber. The region is mountainous and the heavy stands reach to high elevations with few rock outcroppings. According to Silburn (10), 96% of the merchantable timber and all the immature timber are accessible. Western hemlock and fir form the greatest portion of these stands, but a considerable amount of western red cedar (*Thuja plicata* D. Don.) and Douglas fir (*Pseudotsuga taxifolia* (Poir.) Britt.) is also present. Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and western white pine (*Pinus monticola* Dougl. ex D. Don.) occur scattered throughout the region, with yellow cedar (*Chamaecyparis nootkatensis* (Lamb.) Sudw.) at higher elevations.

The four study areas analyzed, indicated in Text-fig. 1, contained an average gross volume of 45,900 bd. ft. of merchantable timber per acre. Each area, based on geographic location, contained a slightly different forest type as follows:

1. The stands were mainly western hemlock and fir, with western red cedar occurring frequently. In the valley bottom and on the slopes, the average gross merchantable volume per acre in mature stands was 42,000 bd. ft., dropping to 41,300 bd. ft. on the hill tops. In the young stands western hemlock predominated with a considerable volume of true fir. In the over-mature stands western hemlock and red cedar were the only merchantable trees, with a thrifty stand of small fir of nonmerchantable pole size frequently observed.

2. Western hemlock and fir predominated in the valley bottom, but at higher elevations fir was not present, and yellow and red cedar became frequent. Gross merchantable volumes averaging 59,600 bd. ft. for mature stands of western hemlock and fir were found in the valley bottoms, dropping to 40,100 on the hill tops.

3. There was less fir here than in the previous types, but Douglas fir was present. The western hemlock and fir together contained a volume of 49,000 bd. ft. gross merchantable volume per acre.



TEXT-FIG. 1. Location of the study areas in the Juan de Fuca forest region. The numbered circles indicate the approximate location of the areas studied.

4. The stands here were made up chiefly of western hemlock and red cedar, with fir rarely present. The former species ran to an average volume of 58,200 bd. ft. per acre gross merchantable volume in mature stands.

## Method of Study

### SAMPLE PLOTS

In 1945 the plots had contained 0.1 acre each, but laying out these plots and analyzing the data obtained was too time consuming for the small areas. Therefore, each group of four adjacent plots was analyzed as one and in 1946 sample plots two chains square, containing 0.4 acre, were laid out mechanically in lines four chains apart. This procedure was varied somewhat to avoid concentrations of species other than western hemlock and fir.

The majority of these plots were established in felled and bucked timber. Where samples were required of a forest area outside felled and bucked timber, the trees were felled and bucked according to the standard practice for the region. To obtain age-diameter relationships and other data, all trees were felled on each sample plot regardless of merchantability.

### FIELD PROCEDURE

All trees on the sample plots were scaled according to the British Columbia Log Rule (9). Each tree was scaled as bucked for merchantable volumes according to the practice of the logging company operating on the area, and to a 10-in. top diameter for standardization of the results. Stump height, diameter at stump height inside bark, diameter at breast height inside and outside bark, and wherever possible, total height of the bole, were recorded for each tree. Notes were kept concerning the position of crooks, large knots, spiral grain, conks, and other visible defects. The location and size of decayed areas were recorded and volume deductions calculated according to standard practice. All data were recorded on U.S. Forest Service form 228A, giving a clear picture of the position and extent of all defects and abnormalities.

The age of each tree was determined at stump height and total age computed by adding to this figure the estimated number of years required for a tree to grow to stump height. This latter was determined by sectioning western hemlock and fir reproduction in adjacent areas. The diameter increment was recorded for each tree by counting the number of rings per inch along the average radius of the stump. Where decay or other defects in the butt made a ring count impossible, the number of growth rings of the destroyed portion was considered the same as that for adjacent trees showing a similar increment pattern.

### COMPILATION OF DATA

A clear picture of each tree sampled with the position and extent of all defects or abnormalities was given by the forms used in the field. Cubic volumes can be obtained from these forms with a planimeter but they have not been given in compiling the results of this study, as there is no standard practice for their use in British Columbia at the present time. Compilation was made of the merchantable board foot volumes (or volumes utilized

according to present practice) and volumes to a standard 10-in. top diameter inside bark unless otherwise stated. All diameter breast height measurements are outside bark.

## Results

### FUNGI CAUSING DECAY IN LIVING TREES

#### Occurrence of Fungi Causing Decay

The major decay organisms were approximately of the same relative frequency in both western hemlock and fir as shown in Table I. The well known

TABLE I

THE OCCURRENCE OF FUNGI CAUSING DECAY IN LIVING WESTERN HEMLOCK AND FIR IN THE FRANKLIN RIVER AREA

Organism	Type of decay	Western hemlock		Fir	
		Number of infections	Percentage of all infections	Number of infections	Percentage of all infections
Root and butt rots		259	51.2	83	41.9
<i>Poria subacida</i>	White spongy	104	20.6	35	17.7
<i>Fomes annosus</i>	White spongy	35	6.9	6	3.0
<i>Armillaria mellea</i>	Yellow spongy	71	14.0	13	6.6
<i>Polyporus sulphureus</i>	Brown cubical	9	1.8	5	2.5
<i>Polyporus circinatus</i>	White pitted	7	1.4	2	1.0
<i>Polyporus Schweinitzii</i>	Brown cubical			1	0.5
<i>Poria Weirii</i>	Brown stringy	11	2.2		
<i>Poria nigrescens</i>		1	0.2		
<i>Poria asiatica</i>	Brown cubical	1	0.2		
Mixed and unknown		20	3.9	21	10.6
Trunk rots		227	44.9	105	53.0
<i>Fomes pinicola</i>	Brown cubical	101	20.0	39	19.7
<i>Fomes Pini</i>	White pitted	46	9.1	2	1.0
<i>Stereum abietinum</i>	Brown cubical	29	5.7	30	15.1
<i>Fomes Hartigii</i>	White spongy	13	2.6	2	1.0
<i>Hydnum</i> sp. ( <i>H. abietis</i> )	Yellow pitted	7	1.4	11	5.6
<i>Fomes applanatus</i>	White spongy	7	1.4		
<i>Polyporus abietinus</i>	White spongy			3	1.5
Mixed and unknown		24	4.7	18	9.1
Sap rots		20	3.9	10	5.1
<i>Fomes pinicola</i>	Brown cubical	12	2.3	5	2.5
<i>Polyporus abietinus</i>	White spongy pitted	6	1.2	4	2.1
<i>Fomes applanatus</i>	White spongy	1	0.2		
<i>Fomes Hartigii</i>	White spongy			1	0.5
Mixed and unknown		1	0.2		
TOTAL		506		198	

root and butt rots caused by *Poria subacida* (Peck) Sacc., *Fomes annosus* (Fr.) Cke. (Plate I), and *Armillaria mellea* Vahl ex Fr. (Plate II), and the trunk rot, caused by *Fomes pinicola* (Sw.) Cke. (Plate III), occurred frequently

in both species. A major trunk rot, caused by *Fomes Pini* (Thore) Lloyd (Plate VI), showed a marked difference in occurrence on western hemlock and fir. Less than 1% of all infections, or two cases of decay, caused by *F. pini* were found on fir in the sample plots investigated. *Polyporus Schweinitzii* Fr., the cause of a brown cubical butt rot of frequent occurrence in many tree species, did not occur on western hemlock on the study area, and was found only once on fir.

The frequent occurrence of *Stereum abietinum* Pers. (Plate IV), causing a brown cubical pocket rot of both western hemlock and fir, is of much interest. This fungus has not been reported previously as an important decay organism of either tree species. *Hydnum* sp. (*H. abietis*) (7) occurred so frequently in living western hemlock and fir that it can be classified as the cause of a major decay of both species. This is the same fungus as that referred to as *Hydnum abietis* Hubert by Englerth (6), who reported it as the cause of a minor decay in western hemlock.

*Poria nigrescens* Bres., reported as causing decay in living Sitka spruce (2), was found in living western hemlock but not in fir. *Poria asiatica* (Pilát) Overh., reported as an important decay of western red cedar (5), was also found in western hemlock but not in fir.

*Ganoderma oregonense* Murr., reported by Englerth (6) as causing decay of western hemlock, was observed on this species in the area under investigation, but did not occur on any of the sample plots. *Echinodontium tinctorium* Ell. and Ev., reported on western hemlock and fir by Bier (1, 3), Englerth (6), and others, was not observed anywhere in the region.

#### *Avenues of Entrance of Infections*

As the avenues of entrance of infections might on occasion be used as an external indication of decay, they are worthy of attention. During the course of this study there were 338 cases where these entrances were noted. These were all either roots, scars, branch stubs, or broken or dead tops. Table II lists the organisms responsible for the decays noted in this study and their place of entrance.

It is interesting to observe that of the 338 infections where the avenue of entrance was recorded, 198, or approximately 60%, entered the trees through scars. As would be expected, a great number of the root and butt rots entered through the roots, but an even greater number of this group entered through scars. Of the trunk rots the majority, 58%, entered through scars and 36% through branch stubs. These figures stress the great number of infections that gain entrance through scars. As the majority of these scars were caused by falling trees or snags, the danger of partial or selective logging in hemlock-fir stands is indicated.

#### *Relative Importance of Fungi Causing Decay*

Comparison of the relative importance of the fungi causing decay in living western hemlock and fir is shown in Table III. This table shows only those



TABLE II

AVENUES OF ENTRANCE OF INFECTIONS IN WESTERN HEMLOCK AND FIR IN THE FRANKLIN RIVER AREA

(BASED ON 338 CASES OF INFECTION)

Organism	Total number of infections	Avenues of entrance			
		Roots	Scars	Branch stubs	Damaged tops
		Number of infections			
Root and butt rots	168	74	91	3	
<i>Poria subacida</i>	79	31	46	2	
<i>Fomes annosus</i>	13	6	7		
<i>Armillaria mellea</i>	56	32	24		
<i>Polyporus sulphureus</i>	5	1	3	1	
<i>Polyporus circinatus</i>	9		9		
<i>Poria Weirii</i>	5	3	2		
<i>Poria asiatica</i>	1	1			
Trunk rots	119	2	70	43	4
<i>Fomes pinicola</i>	45	2	29	12	2
<i>Fomes Pini</i>	33		21	12	
<i>Stereum abietinum</i>	21		10	11	
<i>Fomes Hartigii</i>	7		1	5	1
<i>Hydnum</i> sp. ( <i>H. abietis</i> )	5		3	2	
<i>Fomes applanatus</i>	6		4	1	1
<i>Polyporus abietinus</i>	2		2		
Sap rots	20		19	1	
<i>Fomes pinicola</i>	15		14	1	
<i>Polyporus abietinus</i>	3		3		
<i>Fomes applanatus</i>	1		1		
<i>Fomes Hartigii</i>	1		1		
Mixed and unknown	31	13	18		
TOTALS	338	89	198	47	4

organisms responsible for more than 5% of the total volume of wood lost through decay in either species. While the eight major decay organisms listed in Table III were not of the same relative importance in both species of trees, the percentage of the total volume of decay caused by these organisms, over 80%, was almost identical. These decays, together with mixed and unknown rots, accounted for 90% of all volume loss from decay.

The root and butt rots (Plates I and II), while responsible for decay in approximately 50% of all infected trees (Table I), were only responsible for approximately 25% of the volume loss from decay (Table III). This indicates that these rots are restricted primarily to a few feet in the butt, while the trunk rots (Plates III to VII) frequently grow considerable distances through the bole of the infected tree.

*Fomes pinicola* (Plate III), causing 40% of the decay in both species, appears to be responsible for the major loss in both western hemlock and fir.

TABLE III

THE RELATIVE IMPORTANCE OF FUNGI CAUSING THE MAJOR LOSS THROUGH DECAY IN WESTERN HEMLOCK AND FIR IN THE FRANKLIN RIVER AREA

Organism	Volume of decay			
	Western hemlock		Fir	
	Percentage of total infections of all trees	Percentage of total volume of decay	Percentage of total infections of all trees	Percentage of total volume of decay
Root and butt rots				
<i>Poria subacida</i>	1.4	10.2	0.8	11.5
<i>Fomes annosus</i>	1.0	7.3	Trace*	Trace
<i>Armillaria mellea</i>	0.9	6.4	0.3	4.7
<i>Polyporus sulphureus</i>	0.1	0.8	0.4	5.7
Totals	3.4	24.7	1.5	21.9
Trunk rots				
<i>Fomes pinicola</i>	5.8	40.8	2.9	40.5
<i>Fomes Pini</i>	1.8	12.8	Trace	1.3
<i>Stereum abietinum</i>	0.4	3.0	0.4	6.2
<i>Hydnum sp. (II. abietis)</i>	0.2	1.3	0.9	12.1
Totals	8.2	57.9	4.2	60.1
TOTALS	11.6	82.6	5.7	82.0

\* "Trace" indicates that the percentage of rot was less than 0.1.

*Poria subacida* (Plate I), *Armillaria mellea* (Plate II), and *Stereum abietinum* (Plate IV) caused similar damage in both species, while the other organisms varied appreciably in relative importance. *Fomes annosus* (Plate I) and *Fomes Pini* (Plate VI), while occurring to a very limited degree in fir, were of importance in western hemlock. The converse was true, however, in the case of *Polyporus sulphureus* Bull. ex Fr. (Plate III) and *Hydnum sp. (II. abietis)* (Plate V). While there are several instances of different relative importance of specific decays in the two species of trees, the whole decay picture for both western hemlock and fir is remarkably similar.

#### DECAY IN RELATION TO AGE

Rather than depicting the condition of a stand at each period of its life, the results of this study give a picture of the condition of each tree of a given age in the stand. In computing maximum net periodic increment correctly, the life cycles of the trees on a single area throughout a complete rotation should be observed. It is evident, however, that such a method is not practicable. The only feasible method known at present of obtaining data to work out a relationship between decay and age in uneven-aged mature stands is to

study individual trees. The use of such a basis in the mature and over-mature stands investigated gives erroneous results as the trees that did not survive early competition and disease, recent losses being indicated by the presence of snags, are of necessity omitted. It can also be seen that this method of carrying out a study on the individual tree basis will give too high a maximum net periodic increment.

### *Incidence of Decay*

It is indicated in Table IV, which outlines the relation between age and incidence of decay in western hemlock and fir in the Franklin River area,

TABLE IV

THE RELATION BETWEEN AGE AND INCIDENCE OF DECAY IN WESTERN  
HEMLOCK AND FIR IN THE FRANKLIN RIVER AREA

Age class	Percentage of trees with decay	
	Western hemlock	Fir
51 - 100	8.2	0.0
101 - 150	23.9	11.1
151 - 200	30.9	15.1
201 - 250	36.7	27.0
251 - 300	44.2	28.8
301 - 350	58.6	47.6
351 - 400	68.8	88.9
401 - 450	85.1	100.0
451 - 500	86.0	100.0

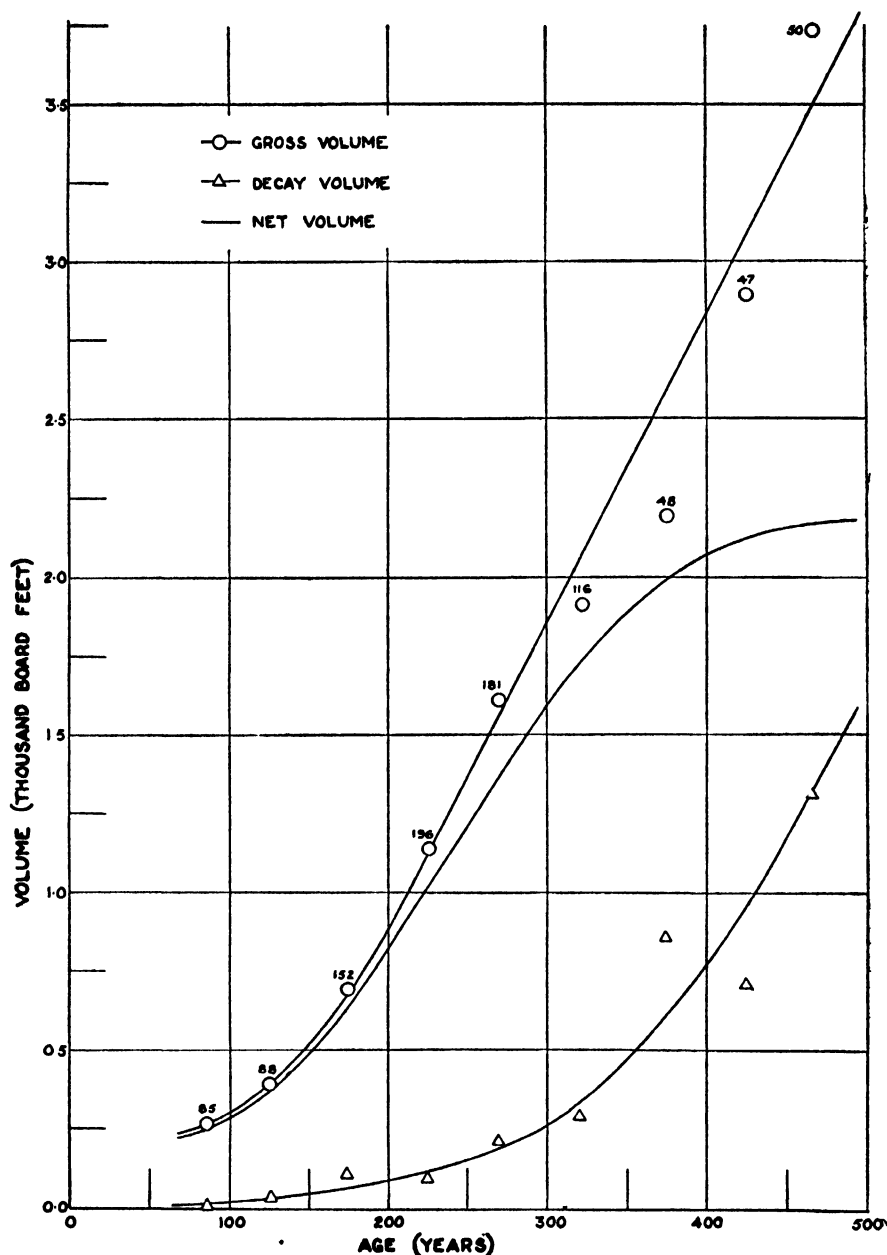
that fir is a shorter-lived tree than is western hemlock. While all fir over 400 years of age were infected, it was found that this condition was not true of western hemlock in the 500-year age group covered by the study.

It is evident from Table IV that (up to 350 years of age) fir is more resistant to infection than is western hemlock. The resin pockets present in the bark of the former may account for this to some extent. The resin forms a coating that probably prevents the entrance of decay organisms through scars and wounds, the main avenue of entrance for wood-rotting fungi (Table II).

### *Losses Through Decay*

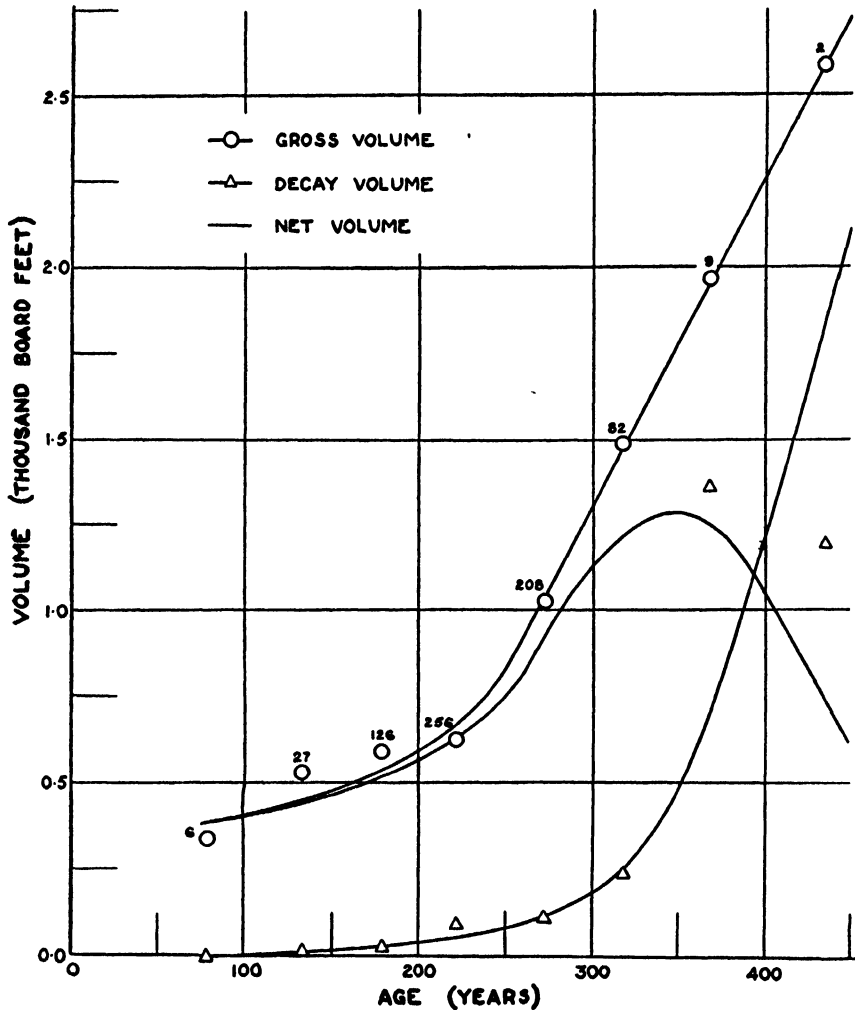
The average gross board-foot volume, the volume of decay, and the average net volume on age for western hemlock and fir are shown in Text-figs. 2 and 3 respectively. The curved values computed from these graphs are given in Tables V and VI.

Western hemlock (Text-fig. 2 and Table V) reached a maximum gross periodic increment of 490 bd. ft. per tree from 225 to 275 years of age. This increment did not vary in the 50-year periods from 275 to 525 years. During the 225- to 275-year period the maximum net periodic increment of 400 bd. ft.



TEXT-FIG. 2. Relationship between age and gross volume, decay volume, and net volume, to a 10-in. top, in western hemlock in the Franklin River area.

was also reached with a decay loss of 13.0%. For the 50-year periods from 175 to 325 years, the net periodic increment was within 50 bd. ft. of the maximum. Following the peak net growth (225 to 275 years of age), the net periodic increment decreased to 60 bd. ft. for the period from 475 to 525



TEXT-FIG. 3. Relationship between age and gross volume, decay volume, and net volume, to a 10-in. top, in fir in the Franklin River area.

years. As the net periodic increment never became a negative quantity, the average net volume gained continually, being 230 bd. ft. per tree at 75 years of age and increasing to 2180 bd. ft. at 475 years of age.

Fir (Text-fig. 3 and Table VI) reached a maximum gross periodic increment of 480 bd. ft. per tree from 275 to 325 years of age. As with western hemlock, this increment did not vary throughout the remainder of the periods studied. The maximum net periodic increment of 310 bd. ft. was reached during the same period as the gross increment. While the 50-year period preceding this peak showed an increment of 300 bd. ft., the period following it showed a loss of 30 bd. ft. net. This sudden drop is further emphasized in the average

TABLE V

THE RELATIONSHIP BETWEEN AGE AND VOLUME OF DECAY AND NET PERIODIC INCREMENT  
IN WESTERN HEMLOCK IN THE FRANKLIN RIVER AREA

Age	Number of trees	Average gross volume, bd. ft.	Average decay volume, bd. ft.	Percent-age of decay	Average net volume, bd. ft.	Average gross periodic increment, bd. ft.	Average net periodic increment, bd. ft.
75	85	240	10	4.2	230	160	140
125	88	400	30	7.5	370	290	260
175	152	690	60	8.7	630	430	370
225	196	1120	120	10.7	1000	490	400
275	181	1610	210	13.0	1400	490	350
325	116	2100	350	16.7	1750	490	240
375	48	2590	600	23.2	1990	490	130
425	47	3080	960	31.2	2120	490	60
475	50	3570	1390	38.9	2180		

TABLE VI

THE RELATIONSHIP BETWEEN AGE AND VOLUME OF DECAY AND NET PERIODIC INCREMENT  
IN FIR IN THE FRANKLIN RIVER AREA

Age	Number of trees	Average gross volume, bd. ft.	Average decay volume, bd. ft.	Percent-age of decay	Average net volume, bd. ft.	Average gross periodic increment, bd. ft.	Average net periodic increment, bd. ft.
75	6	390	0	0.0	390	60	50
125	27	450	10	2.2	440	90	70
175	126	540	30	5.6	510	160	130
225	256	700	60	8.6	640	360	300
275	208	1060	120	11.3	940	480	310
325	82	1540	290	18.8	1250	480	-30
375	9	2020	800	39.6	1220	480	-380
425	2	2500	1660	66.4	840		

net volumes. Starting from 390 bd. ft. per tree at 75 years of age, fir reaches a maximum net volume of 1250 bd. ft. at 325 years of age. The next 100 years shows a decline to 840 bd. ft.

## DECAY IN RELATION TO DIAMETER

The true value of the timber in a stand is obtained from its net volume, i.e., the gross volume minus the volume of decay and natural defects. The gross volume is obtained by cruising a sample of trees in the stand on a basis of diameter at breast height. As few of the internal decays show external indications (page 328), they must be classified as hidden defects. It would be desirable, therefore, to obtain a relationship between diameter breast height and cull for decay for the purpose of estimating net volume of stands. As decay is dependent on age rather than diameter, it is necessary that a close relationship exist between diameter and age before diameter can be used as a basis for estimating decay.

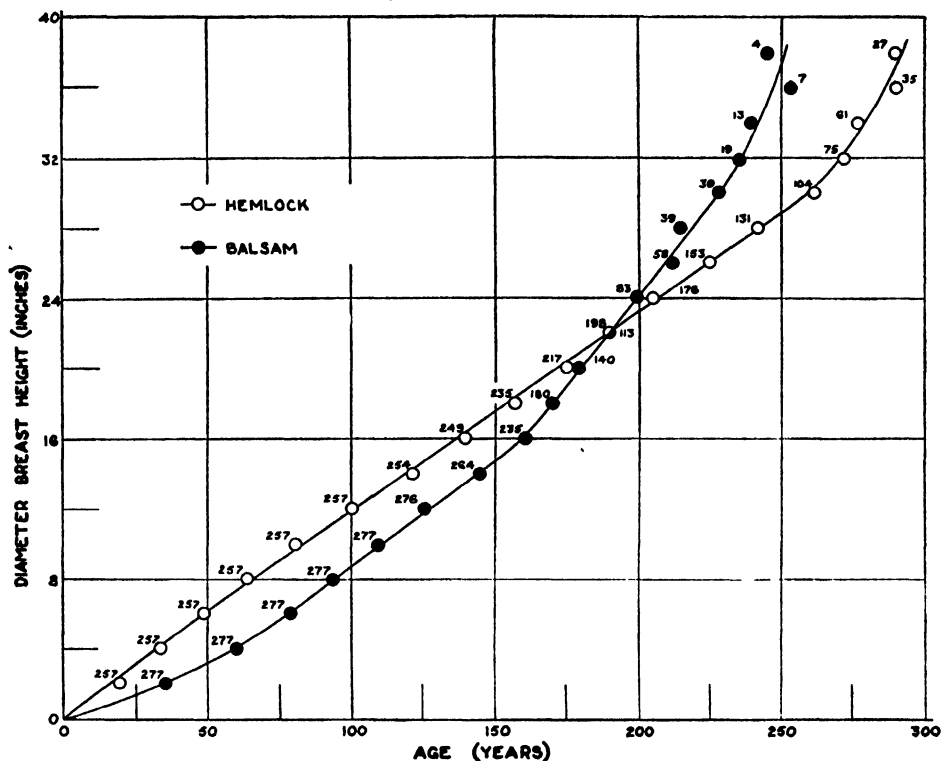
A close relationship is found to exist between age and diameter breast height for both species hemlock and fir in the Franklin River area (Text-fig. 4). The relationship of diameter to age suggests the succession of these two species in the stand. It appears that fir is the less tolerant of the two species as it suffers from suppression during its period of establishment in the stand, while western hemlock is unaffected. The growth rate of fir increases until it is equal to that of western hemlock from 60 to 160 years of age. The opening up of the stand following this period stimulates the growth of the former more than that of the latter, and at 190 years of age the two species have the same average diameter.

Western hemlock in the area shows remarkable uniformity in diameter growth over the first 250 years, reaching approximately 28 in. diameter breast height by that time. Following this the increase in diameter growth is probably indicative of stimulation of growth with some opening up of the stand.

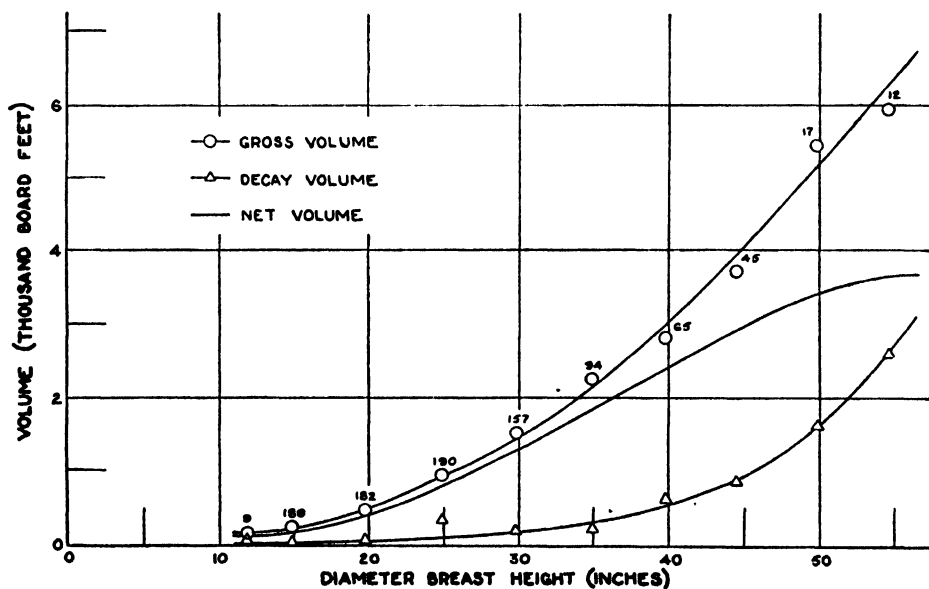
In uneven-aged stands a considerable variation in diameter at any given age is always present. It is therefore essential that any percentage deduction for decay presented in this study be applied only for a diameter class when a large sample cruise has been done. The deduction percentages would not be accurate for a small sample.

The relation between diameter breast height and volume of decay in the Franklin River area is shown in Text-figs. 5 and 6 for western hemlock and fir respectively. The curved values taken from these figures are given in Tables VII and VIII.

Western hemlock does not attain the maximum net volume of 3700 bd. ft. until a diameter breast height of 55 in. is reached (Table VII). At this diameter 42% of the gross volume of 6410 bd. ft. is lost through decay. The greatest net volume of growth occurs in trees between 40 and 45 in. diameter breast height. Between these two diameter limits 600 bd. ft. net volume is added. From 15 in. diameter breast height, the smallest diameter of merchantable size, up to 30 in., the percentage lost through decay varies only

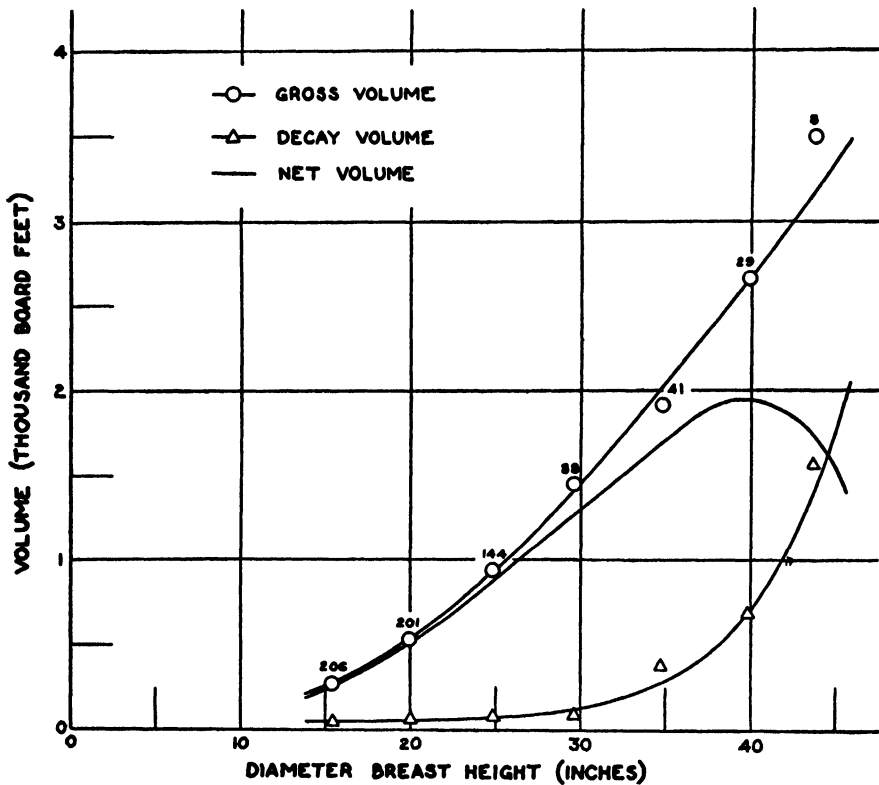


TEXT-FIG. 4. Relationship between age and diameter breast height outside bark in western hemlock and fir in the Franklin River area.



TEXT-FIG. 5. Relationship between diameter breast height outside bark and gross volume, decay volume, and net volume, to a 10-in. top, in western hemlock in the Franklin River area.





TEXT-FIG 6 Relationship between diameter breast height outside bark and gross volume, decay volume, and net volume, to a 10-in. top, in fir in the Franklin River area

from 10 to 13%. Following this there is a gradual increase up to the 45-in. diameter class, after which decay destroys more volume than is gained by an increase in diameter

Fir reaches the maximum net volume of 1900 bd. ft. at 40 in. diameter breast height. Of the 2680 gross board foot volume at this diameter, 29% is lost through decay. The greatest net volume of growth, 400 bd. ft., is added between the 25- and 30-in. diameter classes, but the net volume increase between diameters is fairly uniform from 20 to 35 in. diameter breast height, varying only 30 bd. ft. between 5-in. diameter classes. Trees of a diameter class greater than 40 in. lose considerably more volume through decay than is gained by an increase in diameter.

#### DECAY IN RELATION TO RATE OF GROWTH

To determine whether or not there was a relationship between decay and rate of growth, each tree was placed in a vigor group by the method described by McCallum (8). Trees that fell below an age-volume curve drawn for the whole stand were classified as slow-growing or nonvigorous and those with

TABLE VII

THE RELATION BETWEEN DIAMETER AT BREAST HEIGHT AND VOLUME OF DECAY IN WESTERN  
HEMLOCK IN THE FRANKLIN RIVER AREA

Diameter breast height	Number of trees	Gross volume, bd. ft.	Volume of decay, bd. ft.	Percentage of decay	Net volume, bd. ft.	Net increment between diameter classes, bd. ft.
15	189	280	30	10.7	250	
20	182	530	60	11.3	470	220
25	190	910	100	11.0	810	340
30	157	1490	190	12.8	1300	490
35	94	2190	350	16.0	1840	540
40	65	2980	580	19.5	2400	560
45	45	3950	950	24.1	3000	600
50	17	5120	1670	32.6	3450	450
55	12	6410	2710	42.3	3700	250
60	2	7780	4080	52.4	3700	0

TABLE VIII

THE RELATION BETWEEN DIAMETER AT BREAST HEIGHT AND VOLUME OF DECAY IN FIR IN  
THE FRANKLIN RIVER AREA

Diameter breast height	Number of trees	Gross volume, bd. ft.	Volume of decay, bd. ft.	Percentage of decay	Net volume, bd. ft.	Net increment between diameter classes, bd. ft.
15	206	250	10	4.0	240	
20	201	540	20	3.7	520	280
25	144	950	60	6.3	890	370
30	88	1460	170	11.6	1290	400
35	41	2050	370	18.0	1680	390
40	29	2680	780	29.1	1900	220
45	8	3360	1560	46.4	1800	-100

volumes above the curve were classified as fast-growing or vigorous. With this separation no consistent difference between the two vigor classes at a corresponding age could be found in percentage of volume lost through decay.

### LOSSES OTHER THAN DECAY

During the course of the study, while all computations were made to a 10-in. top diameter for standardization, the actual volumes utilized by the logging company operating in the area were recorded and tabulated as indicated on page 315. Comparison of the average gross board foot volumes utilized to the average gross board foot volumes to a 10-in. top shows that in western hemlock 81% of the volume was utilized, and in fir 84%. For both species the average net volume utilized was 89% of the average net volume to a 10-in. top.

In western hemlock deductions for defects other than decay, such as breakage, crooks, shakes, and splits, in utilized volumes were 78% of those in volumes to a 10-in. top. It is evident, therefore, that as only 81% of the gross volume to a 10-in. top was utilized the majority of cull caused by defects other than decay occurs in the utilized portion of trees of this species. This is probably accounted for by the large amount of ring shake present in the butt logs of mature western hemlock.

In fir, deductions for defects other than decay in utilized volumes were only 53% of those in volumes to a 10-in. top. This would indicate that a considerable proportion of the cull to a 10-in. top for defects other than decay is for breakage.

### Discussion

External indications of decay in the individual tree have, in the past, been the main basis for estimating the loss through decay in a stand. By field observations carried out during this study, it was observed that the fungus, *Fomes Pini*, was the only one that usually produced sporophores or external indications of decay. *Fomes pinicola* frequently produced fruiting bodies indicating decay, but rarely could the extent of decay caused by the fungus be estimated without examining the felled tree. As *F. Pini* caused only 12.8% of all loss through decay in western hemlock and 1.3% in fir, it is evident that decay in such areas can only be accurately estimated by a careful examination of a large sample of felled trees.

The pathological condition of one region cannot safely be assumed to be similar to that of another supporting the same species, as is shown by a comparison of the results obtained by Englerth in western Oregon and Washington (6) and those obtained in this study. Not only were some of the fungi reported by Englerth as causing major losses in decay very infrequent or entirely absent in this investigation, but also the relative losses caused by the different organisms frequently varied appreciably.

As considerable differences in loss through decay occur between two regions, it is also evident that lesser differences, although significant and important, occur between stands on different sites. An attempt was made to separate

sites in this investigation but insufficient sampling made it impossible. Extensive sampling on all sites would be required to make such an investigation complete enough for results to be utilized with accuracy in formulating management plans. A detailed site classification of the region investigated was not available at the time this study was carried out. As no significant differences in amount of decay in relation to age were observed between areas investigated, it is thought that the areas sampled were of a fairly uniform site classification.

### Summary

In the northwestern portion of the Juan de Fuca forest region, between Nitinat Lake and the Alberni Canal on Vancouver Island, the logs of 963 western hemlock and 719 fir were analyzed in detail.

Decay in the former species was found to be caused by 15 organisms and in the latter by 11. The major portion of the decay loss in both hosts was caused by eight species of fungi, those producing the root and butt rots being *Poria subacida*, *Fomes annosus*, *Armillaria mellea*, and *Polyporus sulphureus*, and the trunk rots, *Fomes pinicola*, *F. pini*, *Stereum abietinum*, and *Hydnum* sp. (*H. abietis*).

Scars were the most frequent avenue of entrance for infections. In 59% of the cases of infection studied the fungi had entered through wounds.

Up to 350 years of age fir appears to be more resistant to fungus attack than western hemlock. In the latter maximum periodic volume increment was reached between 225 and 275 years of age, after which a decline occurred. In the former maximum net periodic volume increment was not reached until between 275 and 325 years of age, but following this period volume increment dropped off sharply.

A close relationship between age and diameter was found in both species. Maximum net volume of hemlock was 3700 bd. ft. to a 10-in. top at a diameter of 55 in. In fir this volume was 1900 bd. ft. at 40 in. At the peak net volume of western hemlock 42% of the gross volume was lost through decay, while in fir 29% was destroyed.

In a comparison between fast- and slow-growing trees no consistent difference was found in volume lost through decay.

In western hemlock 81% of the gross volume to a 10-in. top was utilized, and in fir 84%. Losses other than through decay were mostly top breakage in fir and ring shake in western hemlock butts.

### Acknowledgments

The authors are indebted to the Forestry Department of Bloedel, Stewart, and Welch Company for continued and excellent cooperation throughout this investigation. Particular thanks are expressed to Mr. J. S. Mottishaw, Chief Forester, and Mr. E. L. Young, formerly Forester, Bloedel, Stewart, and

Welch Company, for their advice and assistance, and the services of their field parties throughout the course of the study.

Dr. J. E. Bier, Officer-in-Charge of the Dominion Laboratory of Forest Pathology in Victoria during the course of this study, contributed excellent advice and direction. Dr. Mildred K. Nobles, Associate Forest Pathologist, Ottawa, identified from numerous cultures many of the organisms responsible for the decays, rendering an invaluable service in this investigation.

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## EXPLANATION OF PLATES

### PLATE I

*Fomes annosus* and *Poria subacida* in living western hemlock and fir.

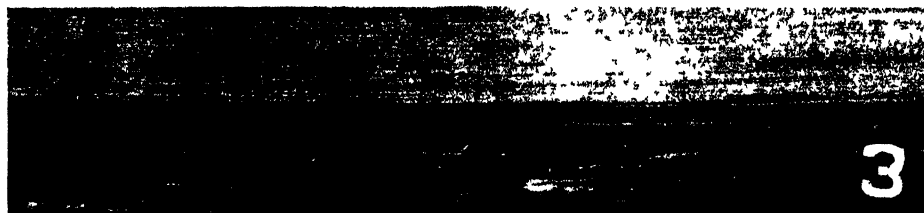
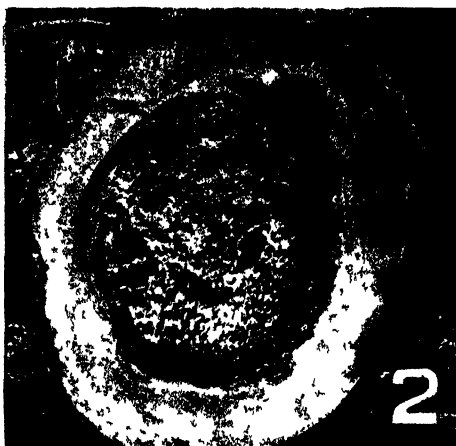
- FIG. 1. Fruiting body of *Fomes annosus* on fir.  
 FIG. 2. Spongy butt rot caused by *F. annosus* in western hemlock.  
 FIG. 3. Spongy butt rot caused by *F. annosus* in fir.  
 FIG. 4. Fruiting body of *Poria subacida*, cracked on drying, on western hemlock.  
 FIG. 5. Spongy butt rot caused by *P. subacida* in western hemlock.

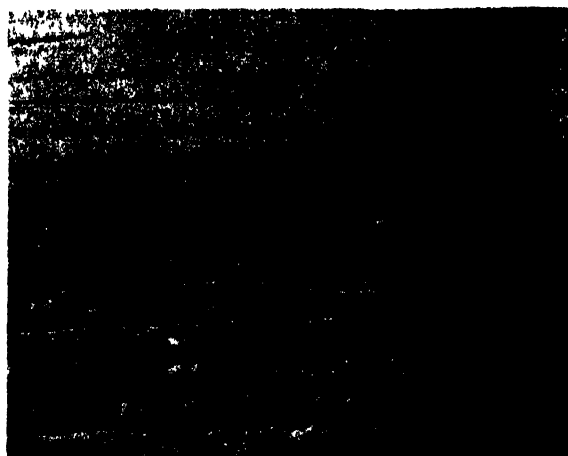
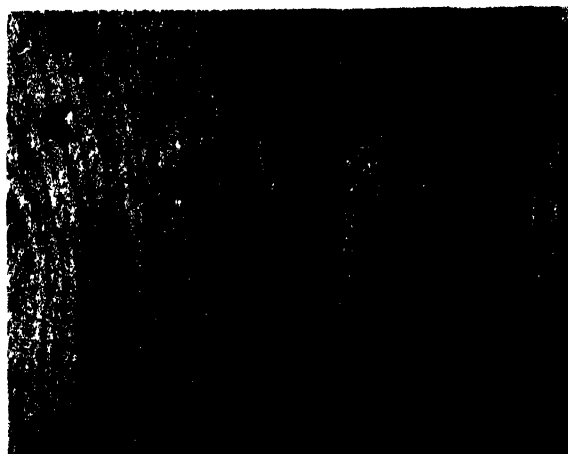
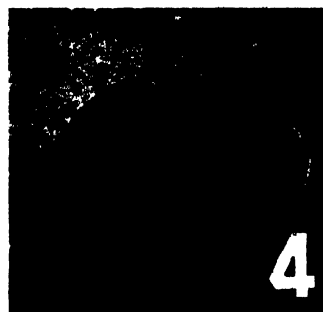
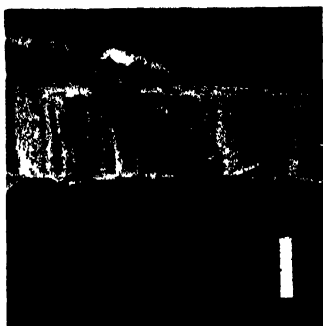
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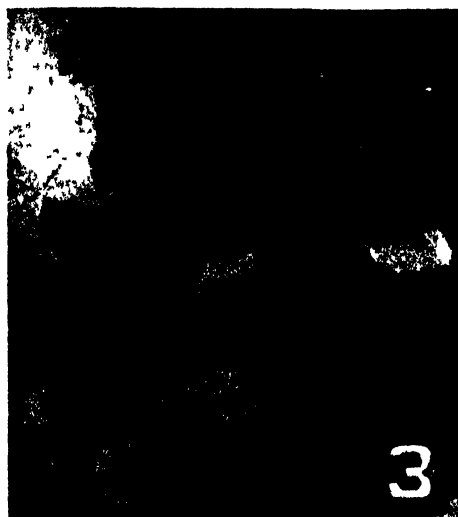
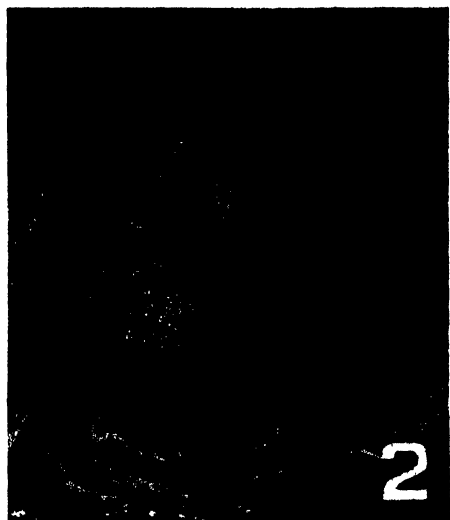
*Armillaria mellea*, *Polyporus circinatus*, and *Poria Weirii* in living western hemlock and fir.

- FIG. 1. Fruiting bodies of *Armillaria mellea* growing from an infected fir stump.  
 FIG. 2. Spongy butt rot caused by *A. mellea* in western hemlock.  
 FIG. 3. Spongy butt rot caused by *A. mellea* with black aerial rhizomorphs in fir.  
 FIG. 4. Fruiting body of *Polyporus circinatus* growing from an infected western hemlock stump.  
 FIG. 5. White pitted butt rot caused by *P. circinatus* in western hemlock.  
 FIG. 6. Decay caused by *Poria Weirii* in western hemlock.  
 FIG. 7. Laminate butt rot caused by *P. Weirii* in western hemlock.

PLATE I









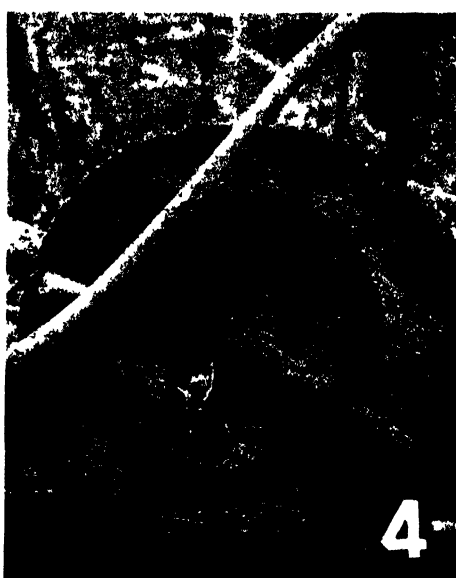
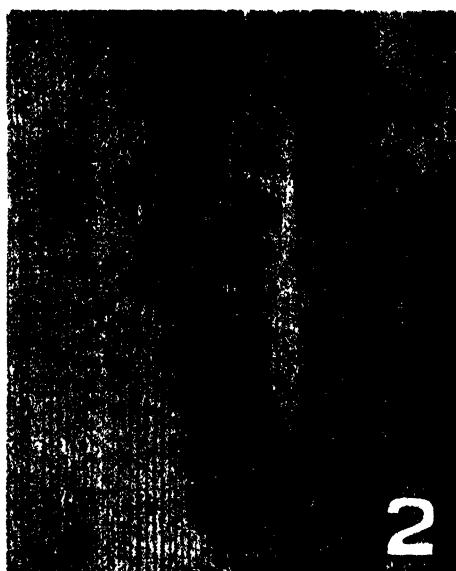
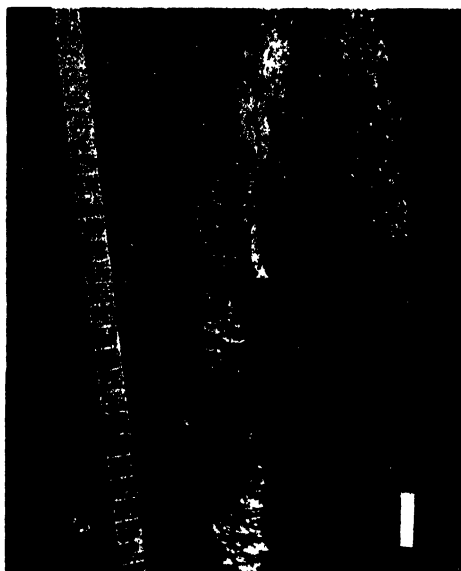


PLATE V

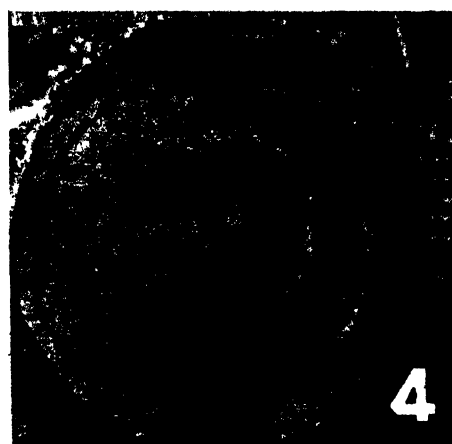
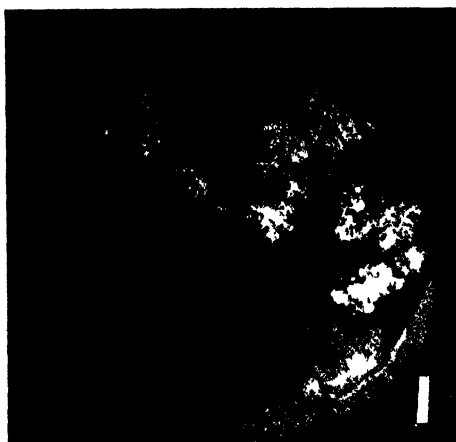
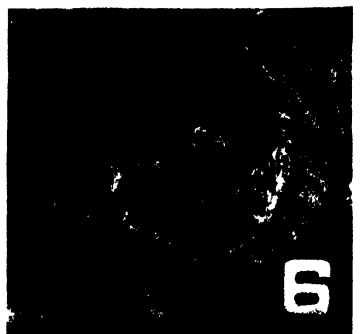
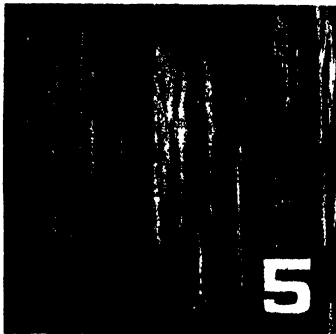
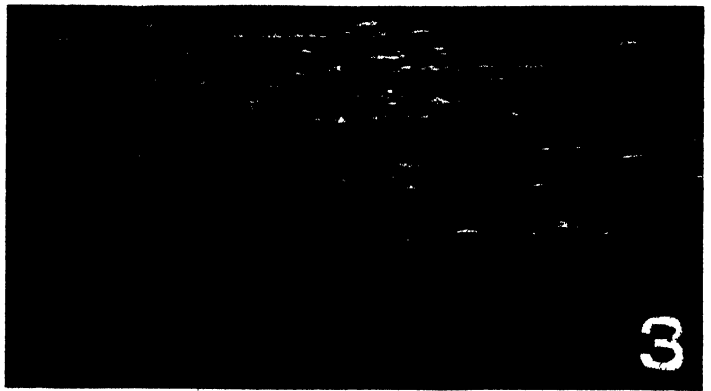
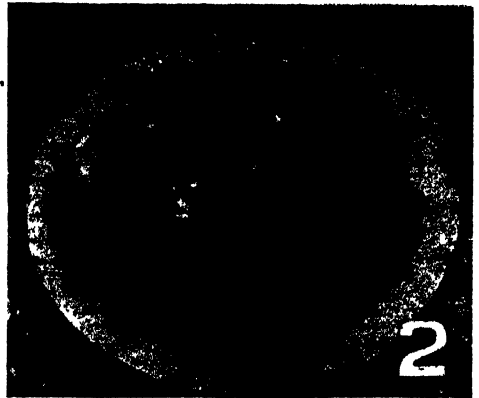
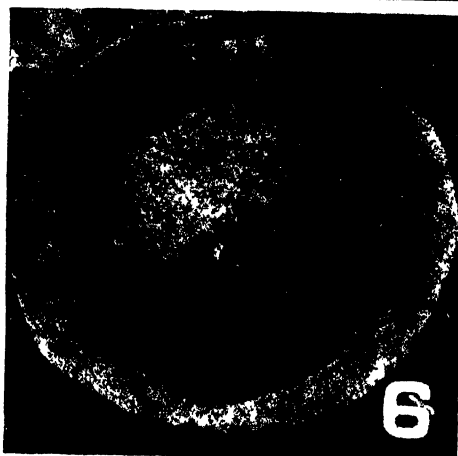
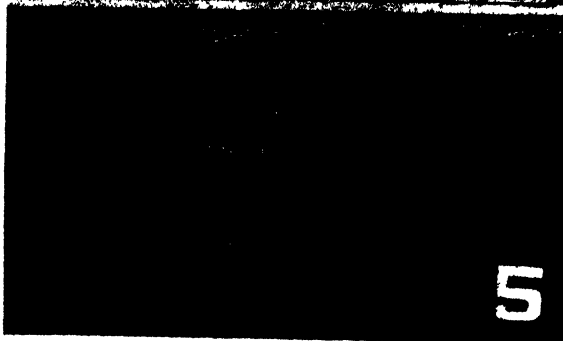
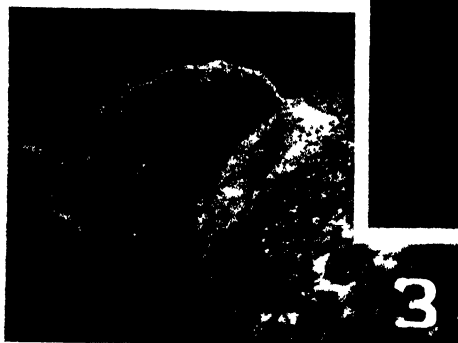
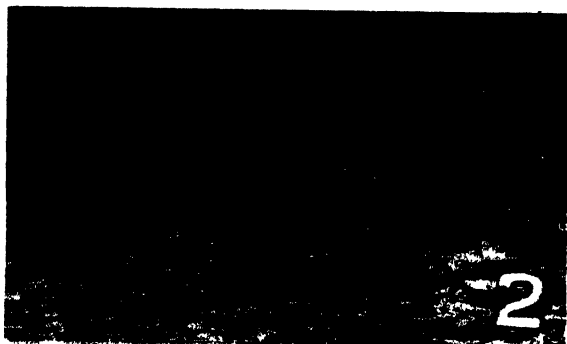
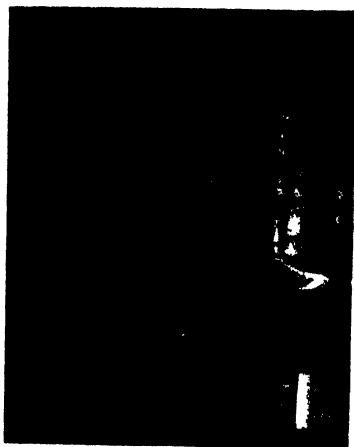


PLATE VI







## PLATE III

*Fomes pinicola* and *Polyporus sulphureus* in living western hemlock and fir.

- FIG. 1. Fruiting bodies of *Fomes pinicola* on western hemlock.
- FIG. 2. Brown cubical trunk rot caused by *F. pinicola* in western hemlock.
- FIG. 3. Fruiting bodies of *Polyporus sulphureus* in western hemlock.
- FIG. 4. Brown cubical decay caused by *P. sulphureus* with white mycelium "felts" in western hemlock.

## PLATE IV

*Stereum abietinum* in living western hemlock and fir.

- FIG. 1. Fruiting bodies of *S. abietinum* growing from a scar on fir.
- FIG. 2. Brown cubical decay caused by *S. abietinum* in fir.
- FIG. 3. Brown cubical decay caused by *S. abietinum* in western hemlock.
- FIG. 4. Brown cubical pocket rot caused by *S. abietinum* in fir.

## PLATE V

*Hydnum* sp. (*H. abietis*) in living western hemlock and fir.

- FIG. 1. Fruiting bodies of *Hydnum* sp. (*H. abietis*) on the end of an infected log of fir.
- FIG. 2. Fruiting bodies of *Hydnum* sp. (*H. abietis*) growing from a standing tree of western hemlock.
- FIG. 3. Yellow pitted trunk rot caused by *Hydnum* sp. (*H. abietis*) in fir.
- FIG. 4. Yellow pitted trunk rot caused by *Hydnum* sp. (*H. abietis*) in fir.

## PLATE VI

*Fomes Pini* and *Polyporus abietinus* in living western hemlock and fir.

- FIG. 1. Fruiting body of *Fomes Pini* on western hemlock.
- FIG. 2. White pitted trunk rot caused by *F. Pini* in western hemlock.
- FIG. 3. White pitted trunk rot caused by *F. Pini* in western hemlock.
- FIG. 4. Fruiting bodies of *Polyporus abietinus* on a small dead fir.
- FIG. 5. Stringy decay caused by *P. abietinus* in fir.
- FIG. 6. Stringy trunk rot caused by *P. abietinus* in fir.

## PLATE VII

*Fomes applanatus* and *Fomes Hartigii* in living western hemlock and fir.

- FIG. 1. Fruiting bodies of *F. applanatus* on western hemlock.
- FIG. 2. White spongy decay caused by *F. applanatus* in western hemlock.
- FIG. 3. Fruiting body of *F. Hartigii* on a felled western hemlock.
- FIG. 4. Fruiting body of *F. Hartigii* on western hemlock.
- FIG. 5. White spongy decay caused by *F. Hartigii* in western hemlock.
- FIG. 6. White spongy decay caused by *F. Hartigii* in sapwood and heartwood of western hemlock.

(Plates I to VII follow.)

# MUTATIONS IN POLYPLOID CEREALS

## I. INTRODUCTORY OUTLINE<sup>1</sup>

BY C. LEONARD HUSKINS<sup>2</sup> AND GERHARD F. SANDER<sup>3</sup>

### Abstract

Fatuoid, steriloid, and subfatuoid oats and speltoid, compactoid, and subnormal wheats arise by mutation which in most cases can clearly be shown to be a chromosome aberration. In all three segregation-types of speltoids, Series  $\alpha$ ,  $\beta$ , and  $\gamma$ , deficiency is definitely established. It ranges from the whole of one particular chromosome, designated C, down to a segment of C too small to be established microscopically, with certainty, at metaphase but still determinable genetically as a deficiency and not a single gene mutation. In the oat mutants there are similar deficiencies but the range apparently extends below the present limit of both cytological and genetic determination. At this point it naturally cannot yet definitely be differentiated from gene mutation, though no evidence favors the latter alternative. In compactoid and subnormal wheats duplication of the whole C chromosome or of its major arm is involved. Natural crossing, particularly between wild and cultivated oats, complicates the problems and is of practical agricultural significance. A distinction can, however, almost invariably be drawn between such hybrids and the mutants. The origin of the mutants and their survival and peculiar genetic behavior are all bound up with the polyploid nature of cultivated oats and wheat. This particularly affects the viability of deficient gametes. The frequency with which different types of gametes are formed is determined by meiotic behavior, which is dependent upon the particular aberrant constitution of the mutant in question; the degree of deficiency or duplication in the gametes affects their selective functioning. All these factors, together with the usual differential between ovules and pollen in the functioning of aberrant gametes, determine the diverse segregation ratios that may be obtained from phenotypically similar mutants.

### Introduction

Offtype plants that resemble other closely related species occur frequently in common oats and wheat, *Avena sativa* L. and *Triticum aestivum* L. (*T. vulgare* Vill.—and herein referred to as such). The commonest of these in oats is the *fatuoid* or false wild oat, so-called because of its resemblance, in diagnostic characters of the spikelet, to the common wild oat *A. fatua* L. Less common are *steriloids* and *subfatuoids*. The former resemble the Mediterranean wild oat, *A. sterilis* L., while the latter, though not like the type of any well recognized "species", resemble specimens that have been included in *A. sterilis*, *A. fatua*, or *A. byzantina* C. Koch. These species all have 42 chromosomes, which, being three times the number found in the simplest diploid *Avena* species, makes them phylogenetically hexaploids though in most respects they segregate like diploids after hybridization. It should be added that the validity of the specific rank given to many forms of *Avena* by Linnaeus, Koch, and others is questionable, though the above-mentioned

<sup>1</sup> Manuscript received in original form June 3, 1948, and, as shortened, August 3, 1949. Contribution from the Department of Botany, University of Wisconsin, Madison, Wis.

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ones at least are very generally referred to as species. In a modern classification, Malzew (35), however, makes all hexaploid *Euavena* members of the two species *A. fatua* L. s. *ampl.* and *A. sterilis* L. s. *ampl.*

In wheat the commonest offtypes are *speloids* and *compactoids* which respectively resemble *T. spella* L. and *T. compactum* Host. The specific rank of these forms also is of doubtful validity by modern taxonomic standards and they also are phylogenetically hexaploids with 42 chromosomes. There are in fact many parallels between the genera *Avena* and *Triticum* in their cytogenetic constitution and their apparent mode of evolution and current variation. For this reason, they are considered together in this introductory outline. In the series of detailed studies which follow in this Journal some of their commonest mutations are analyzed individually. The publication of these papers has been delayed since before the War, but they were summarized meanwhile (23) by the senior author.

A difference of great agricultural significance between the commonly cultivated forms of wheat and oats, and one which affects materially the conditions under which their offtypes are found, is that the threshed "grains" of oats characteristically consist of the caryopsis enclosed in the "flowering glumes", the lemma and palea, while the threshed wheat grain is a true grain or naked caryopsis. The two genera, however, vary alike in this, in kind though not in degree, for *T. spella* "grains" retain their lemma and palea on threshing (as they do in more "primitive" wheat species), while they are lost in threshing *A. chinensis* (Fisch.) Metzger, a cultivated hexaploid oat and also in the "primitive" diploid *A. nuda brevis* Vavilov. It is evidently human fancy that, by selection, has determined the present characteristic difference between oats and wheat in this respect.

In wheat the awn or beard is an extension of the lemma tip while other characters involved in the mutations under consideration are revealed more sharply in the "empty" glumes than in the lemma. In oats, on the other hand, the awn arises from the mid nerve of the lemma just below its tip and the other characters are also predominantly those of the lemma. In view of this difference it is not surprising to find that mutations in wheat may affect the shape and texture, etc., of the empty glumes and the length or presence or absence of awns on the lemma either simultaneously or independently, while in oats the shape, base-type, etc. of the lemma and of the awn on it are usually affected together. Further, associated with the retention of the lemma and palea around the oat caryopsis is the fact that while both awned and awnless wheats are commonly in cultivation, almost all cultivated oats are awnless or have only very weak awns, never twisted geniculate ones such as those of *A. fatua* or *A. sterilis*. Awns on feed oats are always a nuisance, particularly to horses, while awns on wheat are a nuisance only during threshing or in the straw. It is evident, then, that both the phylogenetic development occasioned by human selection and the ontogenetic course of development favor association of awn and lemma characters in oats while freer dissociation would be expected in wheat.



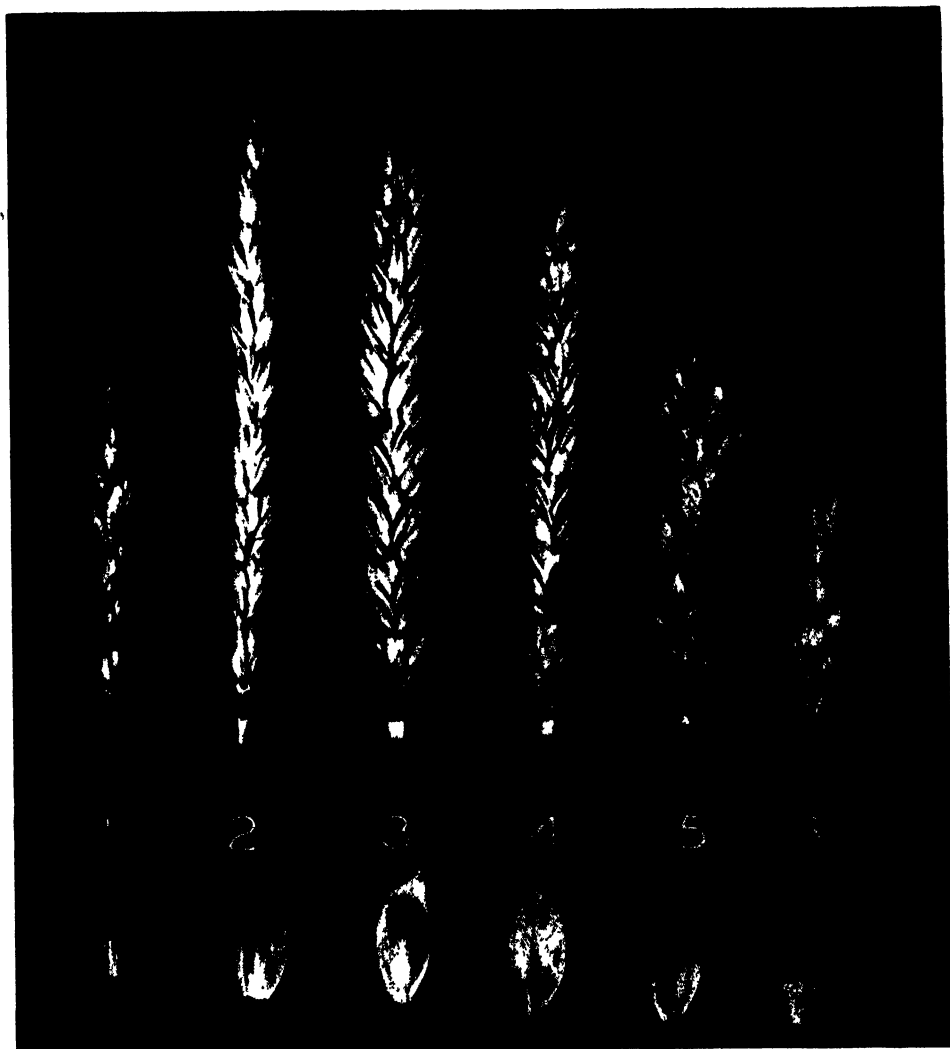
In both genera it is the floral bract characters that are chiefly used in classification of the species. In wheat the shape of the spike is a further conspicuous, though less reliable, character. Most of the other characteristics that differentiate varieties are found within all species of each, though they may differ greatly in degree of development or frequency of occurrence, especially in species that differ in chromosome number. The principal differences between the mutants and "normal" wheat and oats are shown in Plates I and II.

### Historical Outline

Darwin (10) quoted observations of Buckman (5) that apparently mark the beginning of the interest by evolutionists and systematists in the spontaneous variations of oats. Since 1900 both the oat and wheat variations have been the subject of numerous studies by plant breeders and geneticists. Since 1924 there have been many cytological and cytogenetic studies of them. In a recent comprehensive review of the problem (Huskins (23)), 215 contributions to it are cited. Only the most salient features and contributions will be outlined and cited herein.

Nilsson-Ehle (40 and earlier) showed that fatuoids arose in his pure lines of *A. sativa* in an area where there was no *A. fatua* and therefore no chance that they could have resulted from natural crossing with it. His genetic analysis proved them to be mutants. They differ from the variety in which they occur by only one complex of characters of the lemma and this behaves as a unit in inheritance. They are like *A. fatua* in having a strong, twisted, geniculate awn on every grain of the spikelet and a horseshoe-shaped callus ("sucker mouth") or disarticulation surface, surrounded by a tuft of hairs, at the base of each grain. Otherwise they ordinarily differ from any given variety of *A. fatua* in every respect that their parental variety differs from it, though it has been shown by Jones (25) and others that one or two other characters, especially yellow lemma color, may be genetically linked to, or physiologically associated with, the fatuoid complex in some varieties. The mutation almost always appears first in the heterozygous state. This is intermediate in most respects, there being a strong awn on only the primary grain of each spikelet and the base type being nearer *A. sativa* than *A. fatua*. The factors for the cultivated grain type being thus more or less dominant, the heterozygous fatuoid often goes unnoticed in fields of oats and the recessive true-breeding fatuoid that has segregated from it is the form most commonly found.

Tschermak (60 and earlier) has for many years argued that Nilsson-Ehle's and other similar fatuoids arise from natural crosses of *A. sativa* with *A. fatua*, but none of the evidence adduced at various times for this opinion is satisfactory. However, though in heterozygotes or hybrids the dominance of the *sativa* complex is slightly more complete over the mutant than it is over the *fatua* gene complex, the *fatua* and fatuoid complex of genes produce almost, if not quite, identical awns and "sucker-mouths" when homozygous. It is,



Spikes, glumes, and chromosome constitutions in terms of C and Cil chromosomes, of (1)  $\beta$  speloid, (2)  $\beta$  het speloid, (3) normal, (4) subnormal, (5) subcompactoid, and (6) compactoid wheats. Outline figures represent the absence of C. Cil = isomorphic chromosome consisting of a duplicated long arm of C.



therefore, obvious that, from natural crosses with *A. fatua*, segregates may occur that cannot be distinguished from fatuoids that have arisen by mutation unless all characteristics of the plants are considered. Many of the off-type plants found in commercial samples of threshed oats or in fields are the result of natural crossing (Aamodt, Johnson, and Manson (1)). Under optimum environmental conditions, which include cool weather and moisture, most varieties of oats are very largely self-fertilized, but in some areas some varieties have produced as many as 6.6% of hybrid seeds as the result of natural crossing with a different variety planted in adjacent rows (Coffman and Wiebe (9)). The total amount of natural crossing, including that between similar plants which cannot be detected, must, of course, be considerably higher. The amount of crossing varies greatly between varieties and also in different countries. Data for Australia, U.S.A., Canada, U.S.S.R., Scandinavia, and Great Britain may be found in Pridham (47), Coffman and Wiebe (9), Harrington (15), Builin (6), Wexelsen (69), and Jones (26), respectively.

Nilsson-Ehle (38 and earlier) showed that speltoids also are mutants that differ from the variety of wheat in which they occur by only one complex of characters. They also almost always appear first in the heterozygous state. The speltoid "total mutation" from a tip-awned or so-called "awnless" variety (few varieties in general cultivation are truly awnless (Watkins and Ellerton, 68)) involves the appearance of awns and a thickening of the empty glume to a strongly keeled, indurated condition. The "shoulder" of the glume becomes "square" instead of "sloping"; the plant is taller and, correlative, the spike is greatly elongated. Cultivated varieties of wheat differ considerably in these characters and the speltoid characteristics can be described more precisely only in relation to their degree of difference from the variety in which they occur. *T. vulgare* varieties range from very near the type of *T. compactum* to very near that of *T. spelta*. The speltoid mutation is a change towards the latter, the extent of which is determined by its starting point in the characteristics of the particular variety in which it occurs. Heterozygous speltoids are intermediate between the parental normal type and the true-breeding speltoid. There is much less difference in glume characters between speltoids occurring in different varieties or differing in chromosome constitution than between either heterozygous speltoids or the "normals" of different varieties.

Awnless speltoids also arise as mutants from either awned or "awnless" *T. vulgare*, and awned plants, which are otherwise like their parental type, occur in "awnless" varieties. These Nilsson-Ehle has called "part-mutations". The two part-mutations are the result of changes at different loci some 30 or so crossover units apart in one chromosome designated C herein (IX of Sears (55)). The awned speltoid type can be obtained as a crossover segregate

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" Spikelets of: (7) normal, (8) het fatuoid, (9 and 10) steriloid, (11) subfatuoid, and (12) fatuoid oats. Figs. 8 and 10 are var. Banner; 7, 9, 11, and 12 var. Kanota.

from crosses between the two part mutations. Such awned speltoids arising from gene recombination continue to show crossing over between the awn and glume gene complexes while awned speltoids that arise as "total mutations" show no such crossing over. Evidently the total mutation affects both of the genes (or gene complexes) and also the interstitial region between them as Nilsson-Ehle (41) pointed out.

### Genetic Ratios

It was early discovered that phenotypically similar heterozygous speltoids could differ greatly in their segregation ratios. Nilsson-Ehle (39) grouped his strains into three genetic types which he called Series A, B, and C. They will here be called  $\alpha$ ,  $\beta$ , and  $\gamma$  to avoid confusion with chromosome symbols which were introduced later in a cytogenetic study by Winge (70).

Series  $\alpha$  comprises those speltoids whose heterozygous form after self-fertilization produces the normal (vulgar) type, heterozygous speltoids, and speltoids in ratios approaching 1 : 2 : 1. All three classes of segregates are vigorous and fertile. There are usually somewhat fewer speltoids than normals and in some strains the ratio deviates appreciably towards 1 : 1 : few. In "good" Series  $\alpha$  strains normal and mutant gametes are evidently formed in approximately equal numbers and function almost equally well, whether on the male or female side. In strains giving ratios approaching 1 : 1 : few, the evidence indicates that the deviation from random recombination is due to mutant pollen effecting fertilization less often than normal pollen. Both types of ovule apparently function equally well.

Series  $\beta$  heterozygous speltoids produce three or more times as many het speltoid as normal progeny and very few speltoid offspring. These latter are characteristically dwarf and more or less sterile. Evidently Series  $\beta$  het speltoids produce far more mutant than normal male and female gametes but mutant pollen very rarely functions.

Series  $\gamma$  heterozygous speltoids, according to Nilsson-Ehle's original classification, produce slightly more normal than het speltoid offspring and very few speltoids. These rare speltoid segregates are, again, characteristically more or less dwarf and sterile but slightly less so than in Series  $\beta$ . For various reasons, it now seems better to include in Series  $\gamma$  all strains that give ratios close to 1 : 1 : few. There is no sharp dividing line between Series  $\alpha$  and  $\gamma$  in their segregation ratios and this seems to apply to all of their features, including the cytological.

Until 1925 only Series  $\alpha$  was known in fatuoids. Since then all three have been found and also a modified form of  $\beta$  in which the heterozygotes produce normals and het fatuoids in the usual  $\beta$  ratios but also numerous, instead of few, fatuoid offspring, though these are dwarf and sterile as in typical Series  $\beta$ . Evidently the mutant pollen produced by heterozygous fatuoids of modified  $\beta$  functions as well or better than their normal pollen, but the homozygous mutant, besides being dwarfed, produces abortive pollen and is almost completely sterile.

In wheat there is little difference in the frequency with which the three Series have been found. All speltoids are discovered as plants, not as threshed grains, since these lack the diagnostic glumes, and they are usually noticed as heterozygotes as soon as they occur in plantbreeders' plots since they are taller than their parental stock. The frequency of discovery of the mutants is therefore probably a fair indication of the frequency of occurrence of the mutation. This does not hold for fatuoids as it is usually the homozygous segregates that are discovered either in the field or as threshed grains. These are dwarf and sterile in Series  $\beta$  and  $\gamma$ . Heterozygous fatuoids are usually similar in height to their parental stock and they are not very easily noticed in the field. In threshed stocks the grains of the spikelets are separated and the awns broken off. The secondary and tertiary grains of het fatuoid spikelets cannot be distinguished from the normal after they are separated from the primary and the latter are not always strikingly different after the awn is broken off. It is therefore not surprising that most fatuoid strains belong to Series  $\alpha$  whether they are first found as plants or seeds. Series  $\beta$  and  $\gamma$  strains have almost all originated from definite searches for heterozygous fatuoid seeds or plants, though the Series to which they belong cannot be determined from the heterozygous phenotype.

Compactoids usually arise in the heterozygous, intermediate state here designated subcompactoid, and their commonest source of origin is as infrequent and irregular but characteristic segregates from Series  $\beta$ , or more rarely Series  $\gamma$ , het speltoids. They can arise, however, directly from pure lines of normal *T. vulgare*. Subcompactoids that are phenotypically similar may belong to types that segregate very differently. The simplest type, after the customary self-fertilization, gives only normal, subcompactoid and compactoid offspring. Another type gives in addition a small proportion of het speltoids. A third, the commonest, gives normals, subnormals, het speltoids, subcompactoids, and compactoids. The ratios are very irregular and attempts to determine the cause by genetic analyses alone met, not surprisingly, with very little success. They are now known to have complex chromosome constitutions (Håkansson (12, 14); Smith (56, 57); Huskins (22, 23)). Correlatively, their genetic analysis is complicated by much sterility and outcrossing of semisterile plants. In addition there is, as with speltoids only more so, the complication that the types can be classified only in relation to the norm of the variety from which they originated. The subcompactoid arising in a variety of *T. vulgare* with moderately dense spikes and narrow glumes may, for instance, closely resemble in glume and spike characters the compactoid mutant of a long-headed, wide and square-glumed variety while at the same time being very similar to the normal phenotype of a dense-headed, narrow-glumed variety of *T. vulgare* that approaches the type of *T. compactum*.

All of these wheat and oat mutations can, of course, occur either in pure lines or in hybrid stocks. If in the latter, their analysis may be very complex. They can also occur either germinally or somatically. If the latter, the result

is a mosaic plant or chimera. The chimeras found most commonly in wheat are sectorials having one side of the head normal and the other het speltoid. A somatic mutation that produces entire heterozygous mutant culms or tillers, leaving other culms normal, can be discovered only in studies that involve the examination of individual plants and since twin seedlings are not uncommon in wheat (Muntzing (37)) and double oat grains are very common, it is difficult to establish definitely that plants with whole heads of different types are the product of one embryonic zygote. Many chimeras of diverse types have been found by Åkerman (3) and others, including the writers. As the diagnostic characters of all the mutants are maternal tissue, the phenotype of the parent plant does not necessarily give any indication of the genetic constitution of the seeds within the mutant or normal floral bracts. The glumes in wheat are produced by the dermatogen and the seeds by subepidermal layers according to Rosler (48). A periclinal chimera that looked like an ordinary het speltoid but had only the outer cell layer mutated would therefore produce only normal offspring. The ratio of mutant and normal types produced by het speltoid or het fatuoid spikelets or grains of a mosaic spike or panicle rarely gives any indication of the ratio their heterozygous offspring will produce, since the mutation may have affected only the epidermal layer in some of the grains and both the epidermal and subepidermal in others.

### Cytological Interpretations

Nilsson-Ehle classed both fatuoids and speltoids as "loss-mutations" and more specifically, as "complex-mutations" resulting, he thought, from the loss of a "complex" of genes that is necessary for the development of the normal phenotype of cultivated oats and wheat. As early as 1920 he speculated that they might be due to the loss of chromosome segments as, according to the then recent genetic analyses of Bridges, were the "deficiency mutations" of *Drosophila melanogaster*. In this he was remarkably prescient but, speaking generally, though he, Lindhard (31 and earlier), Åkerman (2), and many others proved the mutational origin of the various offtypes and by genetic analyses elucidated many of the peculiarities of the different Series, it was not until correlated cytological and genetic studies were made that the most puzzling features could be clarified and the problem as a whole be understood. The first cytological study was made by Winge in 1924 on various strains obtained from Lindhard and one from Åkerman.

In 1917 Winge had pointed out the very significant role played in plant evolution by chromosome doubling following hybridization, which is now known either as allopolyploidy or amphidiploidy. At that time the chromosome number of wheat had been given as  $2n = 16$  by nine different cytologists. Little was then known of polyploid genetics and, in any case, on the basis of these counts wheat would have been considered a diploid species. Varying numbers up to 44 had been counted in oats. In 1918, it was, however, established independently by Sakamura (49) and Sax (53) that in *Triticum* there are three groups of species with 14, 28, and 42 chromosomes. In 1919

Kihara (29) proved that a parallel situation exists in *Avena*. The cultivated species so far mentioned herein have 42 chromosomes and since these regularly form 21 bivalents at meiosis the cytological presumption is that they are amphidiploids, not autopolyploids that have resulted from chromosome doubling in one species. The results of many genetic analyses of hybrids between the different groups support this (see Sears (54)) and recently Thompson (Thompson, Britten, and Harding (59)) has synthesized a *T. vulgare* type by doubling the chromosome number artificially in a hybrid of *T. turgidum* ( $n = 14$ )  $\times$  *Aegilops speltoides* ( $n = 7$ ). Less is known of *Avena* phylogeny but it has, for instance, been shown by Jones (27) that the awn and lemma characters which are associated in *A. fatua* and fatuoids occur as separately inherited characters in certain diploid and tetraploid oat species. This could be taken to indicate that the geniculate awn and horseshoe base of *A. fatua* grains are due to factors that came into it from two simpler species. We shall see that the mutation data indicate that these same factors are probably present in normal *A. sativa* where their effect is inhibited by a single "cultivated factor" or a closely linked group of factors. When this is lost the fatuoid characters appear.

In his preliminary study of Åkerman's Series  $\alpha$  speltoid strain, Winge (70) found only the normal formation of 21 bivalents in pollen mother cell meiosis. But later in Lindhard's  $\alpha$  speltoids he occasionally found a quadrivalent and 19 bivalents and in heterozygous speltoid sibs there were sometimes 19 bivalents, one trivalent, and an unpaired chromosome. These were taken to indicate the presence of four identical chromosomes in the speltoids and of three identical and one odd chromosome in the heterozygous speltoids. On this basis Winge constructed an ingenious hypothesis. That it is now known to be incorrect in detail does not detract unduly from its value, for it has led the way to a fuller understanding not only of the speltoid and fatuoid problems but of polyploid genetics in general.

Winge pointed out that the amphidiploid set of *T. vulgare* may be assumed to comprise three similar, but not identical, paired sets, or genomes, of seven chromosomes each, which have been derived from three ancestral diploid species. If the three origins are designated A, B, and C, and the chromosomes of a basic set or genome of the genus *Triticum* are numbered 1 to 7, then the 21 pairs of *T. vulgare* may be represented as

$$\frac{1A}{1A} \frac{1B}{1B} \frac{1C}{1C}; \frac{2A}{2A} \frac{2B}{2B} \frac{2C}{2C}; \dots \frac{7A}{7A} \frac{7B}{7B} \frac{7C}{7C}.$$

Only one multivalent chromosome association was seen in the mutants and the genetic analyses indicate that the change producing the mutation has involved only a single factor or one group of linked factors. Therefore only one of the triplicated pairs of chromosomes need be considered; Winge simply dropped the numeral and called it  $\frac{A}{A} \frac{B}{B} \frac{C}{C}$ . Since pure lines and simple Mendelian hybrid ratios occur in *Triticum vulgare*, chromosome A must



normally pair with A, B with B, and C with C. Winge's primary postulate for the origin of speltoid mutants was, however, that B and C may occasionally pair (or the two B's and two C's form a quadrivalent) since, though not identical, they must be phylogenetically similar. At that time meiotic pairing was generally looked on as a measure of general "affinity" between chromosomes that were themselves units. We now emphasize identity of chromosome segments as a primary factor in synapsis during the meiotic prophase and association of whole chromosomes at metaphase as a secondary phenomenon, usually as a result of chiasmata (exchanges of partners) being formed between identical segments when crossing over occurs during prophase. We should now expect the phylogenetically similar B and C chromosomes to have many identical segments but the arrangement of these to be different and some segments to be present in the one that are lacking in the other. To distinguish chromosomes that for phylogenetic reasons have certain segments in common, and are therefore capable of pairing occasionally, from chromosomes that are regular pairing partners or homologues, we may call the former "homoeologues" (Huskins (20)) (homos = the same; homoios = similar).'

If pairing takes place between B and C, then, Winge pointed out, gametes ABB and ACC may be formed. The gamete ABB united with a normal gamete ABC forms the zygote  $\frac{A}{A} \frac{B}{B} \frac{B}{C}$ , which Winge gave as the formula of a Series  $\alpha$  heterozygous speltoid. This was taken to account for its occasional formation of a trivalent, BBB, and a univalent, C. It would, he assumed, segregate normals,  $\frac{A}{A} \frac{B}{B} \frac{C}{C}$ , heterozygous speltoids,  $\frac{A}{A} \frac{B}{B} \frac{B}{C}$ , and speltoids,  $\frac{A}{A} \frac{B}{B} \frac{B}{B}$ , in irregular ratios but approximating 1 : 2 : 1. The four B chromosomes of a speltoid segregate would, of course, form its quadrivalent. The irregularities of pairing in these abnormal chromosome mutants, or *aberranten* as he called them, would be expected to produce other abnormal types of gametes such as ABo and A (BC) C, the former entirely lacking a C chromosome and the latter having a composite (BC) formed by interchange of parts between B and C during the time they were paired.

In Lindhard's "squarehead heterozygote" or "subnormal" type and also in a "perennis" type, which formed a big rosette of leaves and few heads, Winge found only 41 chromosomes. In a subcompactoid he could sometimes count only 41 chromosomes, but in other cells there were 42. He decided that one must sometimes get lost and degenerate in the cytoplasm.

From the point of view of developmental genetics Winge followed Nilsson-Ehle's lead in assuming that the "normal" phenotype of *T. vulgare* is determined by a balance between factors some of which push development towards the spelta and others towards the compactum type. The B chromosomes predominantly carry the former and C the latter factors, Winge assumed.

He expected therefore to find some heterozygous speltoids resulting from the loss of a C chromosome, i.e. of the constitution  $\frac{A}{A} \frac{B}{B} \frac{o}{C}$ . Oddly enough he found none, though this is now known to be the formula of  $\beta$  heterozygous speltoids, to which Series most of Lindhard's stocks belonged. Ironically, it is the only formula of the 14 which Winge gave that now seems to be thoroughly substantiated. In most of the other mutant types, as will be shown in later papers of this series, structural changes have taken place in the C chromosome. In others there are changes in number or balance but apparently not of the precise type that Winge assumed.

Goulden (11) and Huskins (17) had independently begun cytogenetic analyses of fatuoids very shortly after Winge began his cytological study of speltoids. Goulden found that in dwarf fatuoids which would now be classified as modified Series  $\beta$ , the pollen mother cell meiosis was grossly abnormal. He suggested that "at least part of a chromosome which also carries the cultivated factor has somehow been lost". Huskins (18) adopted Winge's interpretation for Series  $\alpha$  fatuoids. He found  $\beta$  and  $\gamma$  strains, hitherto unknown in oats, and determined that the former lack a C chromosome; he thought erroneously — see later — that the latter had an excess of B chromosomes. In a subsequent study of speltoids (19) a similar interpretation for the three Series was given.

Nilsson-Ehle (41) pointed out weaknesses in Winge's hypothesis that were revealed by his own genetic studies of "part-mutations", i.e. beardless speltoids and bearded normals.

Håkansson (12, 13), Muntzing (36), Phipps and Gurney (46), and Uchikawa (64) have confirmed Huskins' observation that Series  $\beta$  het speltoids are monosomics — lacking one C — and that the dwarf speltoids segregating from them are nullisomics — lacking both C chromosomes. Nishiyama (42) has confirmed this for modified  $\beta$  fatuoids. Vasiliev (65) reported the same constitution for a  $\gamma$  speltoid strain studied genetically by Philiptschenko (45) but further analysis of it by S. G. Smith and the present authors have shown that the monosomic and nullisomic plants examined by Vasiliev must have been  $\beta$  segregates. These are produced in irregular numbers by all  $\gamma$  strains and, as shown in the second paper of this series, they occur more frequently than usual in this strain. Final confirmation that loss of the C chromosome produces speltoids has come in the recent analysis by Sears (55) of 17 wheat monosomics and their corresponding nullisomic types. The C chromosome is his number IX.

In one  $\gamma$  het speltoid examined in his original study (19), Huskins observed a heteromorphic bivalent but thought it was not involved in the determination of the speltoid characteristics. In many pollen mother cells of both het speltoids and speltoids he could find only the normal chromosome number but in others there appeared to be 43 and 44, respectively. These higher numbers were taken to be the correct ones (as with the techniques then in use counts

of the entire complement were difficult) and by the same extension of Winge's hypothesis as used for  $\gamma$  fatuoids (18), the constitutions of het speltoids and speltoids of Series  $\gamma$  were given as  $\frac{A}{A} \frac{B}{B} \frac{C}{B}$  and  $\frac{A}{A} \frac{B}{B} \frac{B}{B} \frac{C}{C}$ , respectively.

Muntzing (36), Håkansson (12), and Bynov (7) also recorded 43 and 44 chromosomes in other  $\gamma$  strains. Very soon, however, Huskins (21, 22) discovered that it was the heteromorphic bivalent that was significant and that the counts of 43 and 44 chromosomes were in some cases erroneous and that in others they resulted from the presence of extra chromosomes having little or no effect on the speltoid characters. Shortly afterwards Nishiyama (43, 44) and Uchikawa (61, 62) independently found that  $\gamma$  fatuoids and speltoids characteristically have a heteromorphic bivalent but the normal number of chromosomes. It is now clear that  $\gamma$  speltoids and fatuoids arise through loss of part of the C chromosome. This is most readily detectable in heterozygotes at the first meiotic metaphase when the deficient C is paired with a normal C forming a heteromorphic bivalent. The deficiency varies greatly in size in different strains, sometimes with correlative genetic results in the segregation.

Extensive cytological studies of  $\alpha$  fatuoids and speltoids were made by Nishiyama (42) and Uchikawa (64). They found multivalent associations but concluded that these bore no relation to the fatuoid or speltoid characters. Though they agreed with Huskins that Series  $\beta$  and  $\gamma$  arise through whole chromosome loss and segmental deficiency respectively, they decided that Series  $\alpha$  strains originate by gene mutation. Jones (24) also favored this interpretation, but later (27) favored the assumption of a small deficiency in chromosome C as postulated by Muntzing (36) and by Huskins (22) after his discovery of variable-sized deficiencies in Series  $\gamma$ . On this later interpretation the speltoid or fatuoid phenotype of any Series arises through the loss of "cultivated factors" or "wild-type inhibitors" while the extent of the deficiency determines their diverse segregation (whole chromosome deficiency gives  $\beta$ , large segment  $\gamma$ , and minute segment  $\alpha$ ). Detailed cytological and genetic evidence for this will be presented in subsequent papers of this series.

Since the phenotype of *T. vulgare* is the result of a balance between factors with opposing tendencies there is nothing to be said, on *a priori* grounds, against the hypothesis that speltoids or fatuoids may arise through duplication or substitution as originally postulated for  $\alpha$  and  $\gamma$ , instead of by deficiencies. Sears (55) has found that chromosomes II and XX carry some of the factors postulated by Winge to be on the B chromosome. Continuation of his line of attack on the problem of the contribution of individual chromosomes to the phenotype of *T. vulgare* will give a definite answer. There remains for consideration here the question of the nature and significance of the multivalents found by Winge (70) and Huskins (18, 19) in  $\alpha$  speltoids and fatuoids.

Some of the observations of trivalents and quadrivalents made by the senior author were erroneous, due either to inexperience or to the use of the paraffin-section method now superseded by the squash technique, which is

greatly superior for this material and purpose. Other multivalents, however, were accurately described and their occurrence has been abundantly confirmed by later observations of many workers on diverse  $\alpha$  strains. The problem of interpreting the significance of these in relation to the mutations hinges on their relative frequency. Nishiyama (42) and Uchikawa (64) concluded, as mentioned, that in their  $\alpha$  strains multivalent formation and other irregularities of meiosis were no more frequent in the mutants than in their normal sibs. But comparisons, to be conclusive, must be between a normal variety and het speltoids that have newly arisen from it, for if, as postulated by Winge, the constitution of an  $\alpha$  het speltoid were originally  $\frac{A}{A} \frac{B}{B} \frac{B}{C}$  and pairing therein commonly took place between B and C they would exchange segments and the C chromosomes of an extracted normal would no longer be as restricted in their pairing potentials as they originally were. Nor would the four B chromosomes of the postulated  $\frac{A}{A} \frac{B}{B} \frac{B}{B}$  speltoid segregates be segmentally identical. These are, however, theoretical considerations which are unnecessary for the understanding of the  $\alpha$  genetic ratios (we cannot in any particular case expect to know definitely the mode of origin of the deficiency which determines its cytogenetic characteristics). Further, wheat and oat varieties vary greatly in their regularity of chromosome pairing and the total frequency of irregularities found by either Nishiyama or Uchikawa in any of their material is lower than that observed in some pure varieties and many intervarietal and interspecific oat or wheat hybrids—see Hollingshead (16), Thompson (58), Vasiliev and Kamenik (66), Camara (8), Katterman (28), Love (34), and others. Sapchin (52) reported that one "high yielding pure line of spring wheat has 20-30 per cent and up to 50-60 per cent in some years of pollen mother-cells with disordered arrangement of chromosomes". On the other hand, bivalent formation, and meiosis in general, is usually regular in some varieties and strains of wheat and oats.

The "speltoid" strain in which Huskins (19) found the most abundant and clear-cut cases of multivalent formation was at that time suspected of being a natural hybrid between *T. vulgare* and *T. spelta* or some derivative of this cross. This has since been confirmed by Dr. Å. Åkerman in whose cultures the crossing occurred. Many multivalents and irregularities have been found in other natural hybrids occurring in our fatuoid or speltoid strains. It is interesting therefore that a recent re-analysis of Lindhard's and Winge's papers has led the authors to suspect that the strain most extensively examined by Winge may also have been hybrid. The evidence is as follows:

Lindhard's het speltoids were originally of Series  $\beta$ , giving about one normal to eight het speltoids and for the first four generations no speltoid offspring. In  $D_4$  a bearded het speltoid arose. It and a beardless sib gave respectively 6 : 44 : 51 and 9 : 63 : 14 normal, beardless het speltoid and bearded speltoid progeny in  $D_5$ . The latter were all vigorous plants, unlike the sterile dwarf speltoids which Lindhard occasionally got in later generations from typical

Series  $\beta$  lines. The beardless het speltoid was taller than any of its sibs. Sixteen of its beardless het speltoid progeny gave a typical Series  $\beta$  ratio, 29 N : 252 H, but one of the latter was again bearded, while 16 bearded speltoid sibs gave 105 bearded speltoid and four beardless speltoids in  $D_6$ . In  $D_7$  two beardless het speltoids gave Series  $\beta$  ratios, but their offspring included one bearded het, one bearded speltoid, and two beardless speltoids while three others gave ratios like those of  $D_6$ , totalling 29 normals, 161 het speltoids, and 49 bearded speltoids. In all generations there was great variability in height and vigor, especially of the het speltoids. In crosses between speltoids of these lines and normal or compactoid types, the het speltoid  $F_1$  plants all gave Series  $\beta$  ratios in  $F_2$  if the speltoid was the pollen parent. The reciprocal cross gave some Series  $\beta$  het speltoids and some that segregated like the  $D_4$  ancestors to give a number of vigorous speltoid offspring.

These data indicate either natural crossing or the presence of extra chromosomes. Certainly these speltoids, which were the ancestors of the plants from which Lindhard sent cytological material to Winge, were not typical  $\alpha$  either in origin or genetic segregation. It appears significant that Winge found no multivalents in an  $\alpha$  strain that he obtained from Åkerman and that Lindhard recorded that his "speltoids" had hard and tight glumes like those of typical *T. spelta*. In a personal communication to the senior author in 1927 he went further and expressed the opinion that there is no difference between speltoid and *T. spelta*. This conclusion is unacceptable (see Watkins (67)) but it would come naturally to Lindhard if the vigorous plants that he considered to be speltoids segregating from self-fertilized  $\beta$  het speltoids were really the offspring of cross-pollinations by either *T. spelta* or derivatives from it.

Compactoid wheat mutants result from duplication of the factors that are deficient in  $\alpha$ ,  $\beta$ , and  $\gamma$  speltoids. Håkansson (12, 14) found two chromosome types. In one the subcompactoid is a trisomic having three C chromosomes and the compactoid segregates from it are tetrasomic for C. In the other it is only the long arm of the C chromosome that is duplicated; this occurs through the formation of an isochromosome, i.e., one having two similar arms. These and several other types with diverse replications of part or all of the C chromosome were found coincidentally in our material (Huskins (22)) and later by Katterman (28) and Uchikawa (63).

Love (33) and Sander (51) have shown that the steriloid and subfatuid mutants arise through alterations in the C chromosome of *A. sativa*. Provisionally they can be considered part mutations of the fatuid complex. The three types behave like a multiple allelic series (Sander (50), Jones (27)) but it is doubtful that they are due to modifications of a single locus.

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studies were continued at King's College, London, and the John Innes Institution. Since 1930 they have been continued as a sideline interest, carried on jointly with research students — especially J. M. Armstrong, E. A. Kerr, R. M. Love, S. G. Smith, and H. G. F. Sander — see (4, 33, 56, 57, 51). From 1931-35 plants were grown at Macdonald College, Que.; from 1937-44 at the Montreal Botanical Garden. The Department of Agronomy, University of Wisconsin, is currently providing facilities for maintenance of stocks and studies of new issues. For these facilities and help and for grants from the National Research Council of Canada, thanks are here expressed.

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# MUTATIONS IN POLYPLOID CEREALS

## II. THE CYTOGENETICS OF SPELTOID WHEATS<sup>1</sup>

BY STANLEY G. SMITH,<sup>2</sup> C. LEONARD HUSKINS,<sup>3</sup> AND GERHARD F. SANDER<sup>4</sup>

### Abstract

The bearded speltoids that are found in beardless varieties of *Triticum vulgare* are usually segregates from heterozygous speltoids that have arisen through mutational loss of two genes, or gene complexes, that normally determine the glume and, to a lesser extent, the lemma and rachis characteristics that predominantly differentiate *T. vulgare* from *T. spelta*, which is possibly one of its ancestors. These "vulgare" genes are epistatic to genes that in their absence determine indurated, sharply and heavily keeled glumes and bearded lemmas closely resembling those of *T. spelta*. The two "vulgare genes" are both on one chromosome ("C" or "IX"); the hypostatic "spelta" or speltoid genes are probably multiple and have not yet been definitely located.

The "vulgare" genes or gene complexes may be lost independently, giving (after segregation) "part-mutations", i.e. beardless speltoids or bearded normals, or they may be lost together, giving the "total mutation" i.e. bearded speltoid. The mutations that produce the "part-mutants" are necessarily segmental changes, but the total mutants may arise through either segmental or whole chromosome loss.

Loss of an entire C chromosome gives a  $\beta$  het speltoid which, on selfing, gives normal, het speltoid, and bearded speltoid offspring in a ratio varying about the mode 1:5:few. These  $\beta$  bearded speltoids are dwarf, sterile nullisomics having only 20 pairs of chromosomes,  $20_{II}-$ , in place of the  $21_{II}$  normal for *T. vulgare*. The characteristic  $\beta$  Series ratio and its variations are mainly determined by the frequency with which the unpaired C of a  $\beta$  het speltoid is left out of the gynospore nuclei during meiosis and by the lower functioning of 20-chromosome pollen. Zygotic elimination plays a minor role.

Deletion of an appreciable interstitial segment, or the whole, of the long arm of C produces  $\gamma$  het speltoids which on selfing give normals, het speltoids, and speltoids in ratios near 1:1:few. If the segment deleted involves both gene complexes the speltoid segregates are bearded and more or less dwarfed and partially sterile. If it does not involve the beard "inhibitor" they are beardless. The  $\gamma$  ratio is determined largely by the lower functioning of pollen carrying the deletion.

Deletion of a segment of C too small to be established definitely at metaphase but genetically determinable as of not less than about 30 cross-over units in length gives  $\alpha$  het speltoids that segregate normals, het speltoids, and bearded speltoids in ratios approaching 1:2:1 and all segregates are of normal size and fertile. Evidently this deletion does not materially affect pollen functioning.

Hybridization and polyploidy have been involved in the evolution of *T. vulgare* and it is the latter which permits the functioning of gametes bearing deficient chromosome complements and the survival of aberrant types that would be unlikely to exist in a diploid species. The hybrid ancestry accounts for quantitative chromosome changes being able to produce forms that closely resemble ancestral or related species, and also for the fact that crosses between varieties of *T. vulgare* or of closely related species can produce forms that may be phenotypically indistinguishable (or nearly so) from the mutants.

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## I. Introduction

The speltoid mutants of *Triticum aestivum* L. (*T. vulgare* Vill.) here analyzed fall into three main segregation types, Series  $\alpha$ ,  $\beta$ , and  $\gamma$ . The basis of classification is a slight modification of the original A, B, C of Nilsson-Ehle (24). The symbolism is changed not because of the modification but to avoid confusion with chromosome and gene symbols. Heterozygous speltoids of Series  $\alpha$  on self-fertilization give normal, het speltoid, and speltoid progeny in ratios approaching 1 : 2 : 1. The ratios deviate from 1 : 2 : 1 in having a greater or lesser deficiency in the het speltoid and speltoid classes. Series  $\beta$  het speltoids produce mainly normal and het speltoid progeny. In different strains and under varying conditions, the ratio of these varies from 1 : 3 to 1 : 8 or more. Speltoid progeny are either rarely produced or rarely survive to maturity, and, if they do, are characteristically dwarf and sterile. Series C het speltoids on Nilsson-Ehle's definition produce slightly more normal than het speltoid progeny while their speltoid progeny are rare and nearly always more or less dwarf and sterile, though they are slightly more numerous, taller, and more fertile than in Series  $\beta$ . None of the strains received as Series C from various sources, including Nilsson-Ehle himself (see Table I), have given more normals than het speltoids under our conditions or those of Uchikawa (36) to whom seeds of Nilsson-Ehle's Series C Strain 26-1295 were sent. We class as Series  $\gamma$  all strains that give het speltoids and normals in ratios closely approaching 1 : 1 while producing few speltoid progeny and these more or less dwarf and sterile. The difficulty of determining the frequency with which the three Series arise directly from normal wheat was discussed in preceding accounts of the history and general features of the problem of recurring mutations in polyploid wheat and oats (11 and 12). In Nilsson-Ehle's cultures prior to 1921, the three Series had each arisen five times as het speltoid mutations from standard varieties or from lines selected from crosses between standard varieties of wheat. In subsequent generations, however, the ratio-type is not necessarily constant. The most frequent change is from  $\gamma$  to  $\beta$ . This is now known to be due to occasional failure of pairing between the segmentally deficient C chromosome, which is characteristic of Series  $\gamma$ , and its normal homologue. This results in the formation of some ovules entirely lacking a C chromosome. One of these fertilized by a normal pollen grain produces a Series  $\beta$  het speltoid. A change from Series  $\beta$  to  $\gamma$  has several times been reported (24, 16, 17), but it has not occurred in our cultures. One way in which it could occur would be by the loss of the longer arm of the univalent C chromosome of a Series  $\beta$  het speltoid through misdivision of its kinomere and combination of the new "Cts" chromosome with a normal C. Many of the changes in ratio-type may, however, be due to cross fertilization which occurs with rather high frequency on plants which form defective pollen.

The complete speltoid or "total-mutation" dealt with herein involves a complex of glume and awn characters, the speltoids being heavily bearded

and the normals "beardless" or, more strictly, "awn-tipped". Two or more linked factors are involved and no crossing over occurs between them in self-fertilized het speltoids or crosses of their speltoid segregates with normals or other types. Hence Nilsson-Ehle in 1921 termed the process "complex mutation", by which he was referring not to complexity of the ratios, etc. but to the fact that the mutation involves a complex of factors. He considered the possibility (now established) that it might be similar to the deficiency mutations then recently discovered in *Drosophila*.

Cytologically, Series  $\beta$  is the simplest type, since its het speltoids result from the loss of one whole C chromosome. Series  $\gamma$  het speltoids result from a gross deficiency in one member of the pair of C chromosomes, giving a clearly heteromorphic bivalent at first meiotic metaphase. Series  $\alpha$  most probably results from a small deficiency but this is difficult to establish cytologically in most strains and the analyses of it are therefore the least satisfactory. The observations on the three series will therefore for purpose of clarity be presented in the order  $\beta$ ,  $\gamma$ ,  $\alpha$ .

## II. Material and Methods

Most of the strains were obtained from Prof. H. Nilsson-Ehle or Dr. Å. Åkerman, Svalöf, Sweden; one  $\gamma$  Series from Prof. Jur. Philiptschenko, Leningrad, and another from Dr. S. J. Wellensiek, Wageningen, Holland; see Table I.

Unless otherwise indicated, all plants described and progenies recorded have been grown in the field in rows six inches apart, with 12 seeds four inches apart in each row. "Germination" records are made soon after the seedlings appear above ground.

Until 1930 most cytological preparations were fixed by Kihara's (Carnoy and Flemming) method, paraffin-embedded, sectioned, and stained with Newton's iodine-gentian violet. Since 1930 the permanent acetocarmine smear method of McClintock has been used. S. G. Smith found in 1936 that it is possible to obtain excellent acetocarmine smears, greatly superior to sections, from the old paraffin-embedded material. This has been valuable for checking earlier observations. The material is run through three changes of equal parts xylol and *N*-butyl alcohol. After two changes in normal butyl alcohol (and bleaching in equal parts hydrogen peroxide and butyl alcohol if the fixative contains osmic acid), it is run down to 70% ethyl alcohol and smear preparations then made.

The following abbreviations are used for the phenotypes: Sp = speltoid, H or Het Sp = heterozygous speltoid; N or Normal = normal appearance for the parental variety regardless of the differences between varieties; SN = subnormal or "square-head heterozygote" of other authors; SC = subcompactoid; Comp. = compactoid.

TABLE I

ORIGIN AND INTERRELATIONSHIPS OF THE SPELTOID AND COMPACTOID WHEAT STRAINS

Series and strain	Donor and year received	Donor's No. and new number	Information from donor			References and relation to other strains
			Originated		Strain or parent stock	
			As	From		
$\beta$ 1	Åkerman 1926	1924-440 26-53/55	Het B	Het A	Line 0715 ex Börsum	Gave SC Strain 3 (35)
$\beta$ 2	Åkerman 1926	1926-497 26-72	Het B in 1926	$F_8$	Extrakolben $\times$ Br. Schlanstedt	Sib of $\alpha$ 1
$\beta$ 3	Åkerman 1926	1924-921 26-67/71	Het/N chimera in 1919	Het B	Landweizen	Åkerman (2) p. 326
$\beta$ 4*	Nilsson-Ehle 1927	26-1292 26-92/94	No information; apparently from same variety as $\gamma$ 4			
$\gamma$ 1	Åkerman 1926	1924-444 26-62/66	Het A	N	0201 $\times$ Börsum	Gave SC Strain 2 (35) Hakansson (6)
$\gamma$ 2	Philipschenko 1930	— 29-226/8	Het C	$F_8$	<i>T. vulgare</i> $\times$ <i>T. compactum</i>	Gave Strain $\beta$ 5
$\gamma$ 3	Wellensiek 1939	— 39-10/13	"Gives 5 N : 4 H : 1 Sp"			None
$\gamma$ 4*	Nilsson-Ehle 1927	26-1295 26-95/97	No information; apparently from same variety as $\beta$ 4			
$\alpha/\gamma$ *	Åkerman 1926	1924-443 25-56/61	Het A	Het "Constant A type"	Schlanstedt $\times$ 0715	S. G. Smith (34)
$\alpha$ 1	Åkerman 1926	1926-493 26-74	Het A in 1926	$F_8$	Extrakolben $\times$ Br. Schlanstedt	Sib of $\beta$ 2
$\alpha$ 2	Nilsson-Ehle 1929	— 29-27/30	Het A	N	<i>T. vulgare</i> var. Iron	None

\* Strains supplied in 1929 to Dr. Uchikawa.

Symbols for chromosomes are: C = the chromosome which ordinarily carries on its long arm the genes which determine the "normal", *vulgare* type of glume and the tip-awned or "beardless" condition—it is chromosome IX of Sears (32); Cd = a C chromosome with a deficiency detectable at metaphase and thereby lacking the above genes; Ctl = the telokinetic long arm of the C chromosome; Cts = the telokinetic short arm of the C chromosome; C $^{\alpha}$  = the mutated C chromosome of Series  $\alpha$  which has a deficiency too small to be established cytologically at metaphase but determinable genetically as about 30 cross-over units in length; Cil = the isochromosome formed by the duplication of the long arm of a normal C chromosome—it is the "Co" or compactoid-forming, "secondary" chromosome of Hakansson (6) and is, of course, equivalent to two Ctl chromosomes in gene content.

## III. Observations

A. SPELTIDS OF SERIES  $\beta$ (a) Strain  $\beta$  1 (Fig. 23)

Three progeny of Dr. Åkerman's Het Sp 1924-440 r4 were collected by C. L. Huskins at Svalöf in 1926. One was a normal which bred true. The other two were classified as het speltoids. One (26-55) gave a fairly typical Series  $\beta$  progeny ratio as did its Het Sp progeny: 91 N, 414 H, 6 Sp, and 1 unclassified dwarf (Table II).

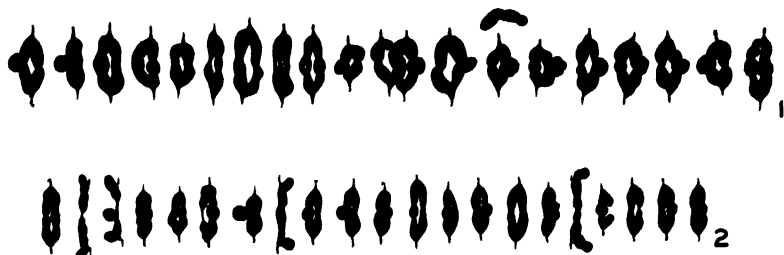
TABLE II

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny				
		No.	Sown		Sown	Germ.	N	H	Sp	Dwf.	Total
1 Original H 26-55	1926	D1	1927	1 —	43	39	8	26	0	0	34
2 H ex H	1927	D2	1928	7 —	396	327	57	255	4 <sup>2</sup>	0	316
3 Resowing	—	—	1930	— 1	3	3	1	2	0	0	3
4 H ex H	1928	D3	1929	3 —	153	109	18	88	2	0	108
5 H ex H	1930	—	1934	1 —	12	12	2	9	0	0	11
6 H ex H	1928	—	1937	1 —	60	26	3	23	0	0	26
7 H ex H	1930	—	1937	1 —	36	17	2	11	0	1	14
8 Total	—	—	—	14 1	703	533	91	414	6	1	512
9 Original N 26-53	1926	D1	1927	1 —	23	22	22	0	0	0	22
10 Total N progeny	'26, '27	D1, 2	'27, '28	5 —	71	63	63	0	0	0	63

The other produced a very varied and, in general, much more vigorous progeny. Both from its segregation and its cytology Huskins (9) concluded that it must have been a hybrid between *T. vulgare* and *T. spelta* instead of a Het Sp. This conclusion was later confirmed by Dr. Åkerman. It evidently arose through fertilization of a normal ovule of Het Sp 1924-440 r4 by a pollen grain from one of the segregates of a cross with *T. spelta* which were being grown in adjacent plots at Svalöf by Dr. Nilsson Leissner. Plant 26-54 and its progeny will not therefore be considered further in the present analysis of Strain  $\beta$  1. Natural crossing between it and a strain intermediate between Series  $\alpha$  and  $\gamma$  gave other complications (34).

In three het speltoids of strain  $\beta$  1 Huskins (9) found a total complement of 41 chromosomes which were "arranged regularly as  $20_{II} + 1_I$ " in pollen mother cell meiosis. Two further het speltoid progeny have since been examined in smear preparations, with the same result (Fig. 1) except that out of many hundreds examined two cells were found with  $19_{II} + 3_I$ . The metaphase shape of the univalent "C" chromosome in this strain has been taken as standard since it seems to be characteristic for most of the  $\beta$  and  $\gamma$

Series strains that we have examined. The univalent C chromosome is divided by the kinomere into two arms, one about four times the length of the other, and the longer arm bears a secondary constriction which divides it



FIGS. 1, 2. Segregates of strain  $\beta$  1:

1. Metaphase I, *het speltoid*;  $20_{II} + C_1$ . Note two bivalents interlocked.
2. Normal segregate with  $21_{II}$ .

into a proximal and distal segment of relative lengths 3 : 5. It is usually found lying either at the periphery of the plate or just off it at metaphase I. As the members of the bivalents pass towards the poles at early anaphase the univalent moves on to the plate. When the remainder of the chromosomes are nearing late anaphase the univalent "splits" and its longitudinal halves proceed to opposite poles but very frequently arrive too late to be included in the daughter nuclei. Deviations from this behavior such as the inclusion of a complete ("unsplit") univalent or of the two halves of a univalent in one dyad nucleus have not been seen in this strain and only rarely in others though genetic evidence that it occurs occasionally is supplied by the occurrence of Type II subcompactoid segregates with three C chromosomes from *het speltoid* parents having only one (35). Nor has transverse fragmentation of the univalent been seen in this strain but there is evidence, in the regular origin of CCil subcompactoids from  $\beta$  *het speltoids*, that it occurs not infrequently and Love (19) has observed it in other wheat mutants. During the second division the "daughter univalents" may be left out of the microspore nuclei or may be included without further "splitting".

Since  $20_{II} + 1_I$  are formed so regularly in  $\beta$  *het speltoids*, the proportion of pollen grains with 20 and 21 chromosomes may be estimated from counts made of micronuclei lying outside the pollen tetrad nuclei. From 150 pollen tetrads it was calculated that 86% of the pollen grains contain only 20 chromosomes — see Section IV.

One normal segregate of strain  $\beta$  1 was found by Huskins (9) to have  $21_{II}$ . One other normal examined since in smear preparations also regularly has  $21_{II}$  (Fig. 2) though out of hundreds of cells examined one was found with  $20_{II} + 2_I$ .

No satisfactory cytological material was obtained from the six dwarf *speltoid* segregates. They would, of course, be expected to have only 40 chromosomes, and we found this in a  $\beta$  *speltoid* derived from a subcompactoid

and repeatedly in the analogous fatuoid segregates. It has also been found by Vasiliev (37), Uchikawa (36), Sears (32), and others.

(b) Strain  $\beta$  2 (Fig. 24)

A second Series  $\beta$  strain (Table III) has been derived from a single newly arisen het speltoid plant, 26-72, found by C. L. Huskins in 1926 in one of Dr. Åkerman's wheat plots. This plot of selected stock (his 1926-497) was

TABLE III

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny				
		No.	Sown		Sown	Germ.	N	H	Sp	Other	Total
1 Original H 26-72	1926	D1	1927	1 —	49	46	14	27	0	0	41
2 H ex H	1927	D2	1928	3 —	108	90	15	74	0	0	89
3 Resowing	—	—	1935	— 2	48	24	4	14	0	0	18
4 H ex H	1935	D3	1936	3 —	105	43	14	26	1	1*	42
5 H ex H	1936	D4	1937	1 —	24	10	1	6	0	0	7
6 Total	—	—	—	8 2	334	213	48	147	1	1*	197
7 N ex H	1927	D2	1928	1 —	12	11	11	0	0	0	11
8 Original chimera 26-73	1926	D1	1927	1 —	22	21	21	0	0	0	21
9 Short-bearded H	1936	D4	1937	1 —	12	5	0	0	0	2*	2

\* Short-bearded H due to natural crossing.

an  $F_2$  progeny from the cross of two standard varieties of *T. vulgare*, Extrakolben  $\times$  Braun Schlanstedt. The short-bearded het speltoid segregate of  $D_3$  had  $20_{II} + 1_I$  but the distal part of the C chromosome seemed to be somewhat broader than in its sib het speltoids and resembled that of strain  $\beta$  4 (Fig. 3 and Fig. 34). Most probably this plant arose through fertilization



FIG. 3. Hybrid het speltoid from  $\beta$  2;  $20_{II} + C_I$  ( $4_{II}$  interlocked).

by a normal pollen grain from one of our Nilsson-Ehle strains, growing in an adjacent bed, in which both the normals and het speltoids are partly bearded.

The Strain  $\beta$  2 het speltoids varied in the shape and tightness of the glumes. Either the parental  $F_2$  selection stock had not yet become homozygous or there had been natural crossing before or after the het speltoid plant was obtained from it.

Five beardless het speltoids all had  $20_{II} + C_I$  and a normal sib had  $21_{II}$ . Cytological material could not be obtained from the single dwarf, sterile, speltoid segregate.

A sib of the original het speltoid, collected at the same time, had only one culm and this was completely het speltoid on one side of the spike but normal in the lower half and het speltoid in the upper half of the other side. Twenty-two seeds from this spike produced 21 normal progeny. The plant was therefore only superficially a chimera.

(c) Strain  $\beta$  3 (Figs. 25 and 26)

This is a winter wheat which has not been grown satisfactorily in Quebec. Progeny ratios and cytological material were obtained from three generations grown in England in 1927-9. Åkerman (2) has described its origin as a chimera occurring in 1919 in the progeny of a het speltoid out of a South Swedish variety. Three het speltoid sibs of the chimera which he propagated from his 1921-924 strain, gave 102 N, 611 H, and 4 weak, sterile Sp progeny. In the next generation, his 1924-921, 10 het speltoids gave 85 N, 334 H, and no speltoid progeny. Of present interest is the occurrence of a fertile and vigorous speltoid among the immediate progeny of the chimera.

One normal, three het speltoids, and one speltoid of this strain were obtained from Dr. Åkerman. The speltoid was a descendant of the vigorous speltoid from the chimera. Sixteen het speltoids have in three generations (Table IV)

TABLE IV

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny				
		No.	Sown		Sown	Germ.	N	H	Speltoids		Total
									Fert.	Ster.	
1 Original H 26-69/71	1926	D1	1927	3	84	45	8	32	0	0	40
2 H ex H	1927	D2	1928	8	223	217	28	181	2	1	212
3 H ex H	1928	D3	1929	5	240	207	24	106	0	0	130
4 Total	—	—	—	16	547	469	60	319*	2	1	382
5 Original N 26-68	1926	D1	1927	1	20	13	13	0	0	0	13
6 N ex N	1927	D2	1928	1	36	34	29	0	0	0	29
7 Total	—	—	—	2	56	47	42	0	0	0	42
8 Original Sp 26-67	1926	D1	1927	1	30	10	0	0	7	3	10
9 Total Sp progeny	'26-'28'	D1-3	'27-'29	8	222	187	0	4	166	7	177
10 N ex H	1927	D2	1928	1	36	33	33	0	0	0	33
11 Total N progeny	'27, '28	D2, 3	'28, '29	2	48	44	43	0	0	0	43
12 Sp ex H	1928	D3	1929	1	36	27	0	0	11	1	12

\* Including one H-N chimera.



given us 60 N, 318 H, 1 chimera, and 3 Sp progeny. The chimera plant bore one chimerical (Fig. 25), two normal, and three het speltoid heads. The three speltoids all occurred in one year, 1928, in a family in which the germination and survival rate was particularly high. One of them was dwarf and sterile, one was of medium height, and one of nearly the same height as the normal sibs. The one of medium height had shorter beards than typical bearded speltoids and produced only a few seeds. Thirty-six seeds from the tallest one produced 1 sterile and 11 fertile speltoids.

Four het speltoids were examined by Huskins (9) and all found to have a typical  $\beta$  complement of  $20_{II} + C_1$ . Smear preparations confirm this.

Four normal plants bred true. Two of them examined by Huskins (9) had  $21_{II}$  excepting for two pollen mother cells, out of about 350 observed, which were thought to contain trivalents. These would probably now be interpreted as bivalents with an unusually large segment distal to unterminalized chiasmata. One apparently true-breeding segregate of normal phenotype examined in a smear preparation made from paraffin material has  $21_{II} + 2$  fragments. The speltoid out of the vigorous fertile speltoid from Åkerman's chimera, together with its descendants produced 4 H, 166 fertile Sp, and 7 sterile Sp progeny. The four het speltoids were almost certainly natural crosses. They gave 6 N, 34 H, and 1 dwarf, sterile Sp; they were Series  $\beta$  as would be expected if they resulted from ovules lacking a C chromosome being cross-fertilized by normal pollen. Åkerman (2) found two such putative hybrids among the 44 progeny of the original speltoid from the chimera.

In one of the speltoids grown in 1927 ( $D_1$  from the speltoid obtained from Åkerman) Huskins (8, 9) recorded the occurrence of 41 chromosomes. He reported, however, that the odd chromosome was not regularly unpaired, as it is in 41-chromosome het speltoids. Trivalents of various shapes were reported to occur in a number of cells. In one cell there were three univalents. From paraffin material of this plant a smear preparation containing 10 good cells has been obtained. The complement is now found to be 41 chromosomes and two fragments. The two fragments were paired in three cells (Fig. 4)



FIG. 4. *Vigorous speltoid from  $\beta$  3;  $20_{II} + 1_I + f_{II}$  ( $f_{II}$  = paired fragments). Note high frequency of interlocking (dissolved paraffin preparation).*

and associated with a bivalent in one; they could not be seen in four and may therefore have been associated with other chromosomes in these cells. In one cell at metaphase, the two fragments and the univalent were all unassociated. In another at anaphase the univalent and one fragment were splitting belatedly—the other fragment had apparently been paired with a bivalent from which it had already disjoined. Re-examination of the original slides of sectioned material has shown that the “three univalents” in Huskins’ (9) Fig. 26 are the univalent and the two fragments unassociated. Some of

the "trivalents" noted in the early records can, after re-examination, still be so interpreted but we hesitate now to make definite decisions about many of the apparently multivalent configurations seen in sectioned material.

(d) Strain  $\beta$  4 (Fig. 27)

This is Nilsson-Ehle's strain 26-1292 of which three het speltoids were obtained in 1926 and given the numbers 26-92/94. The normal and het speltoid segregates are both short-bearded, but there is much variation in number and length of beards in both types. The sterile dwarf speltoid segregates are fully bearded. Twenty-six het speltoids of this strain gave a total of 175 N, 816 H, 3 Sp, and 1 SC progeny besides two dwarfs that did not head (Table V). Five normals gave 160 N and 1 H progeny, the latter an apparent natural cross.

TABLE V

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny				
		No.	Sown		Sown	Germ.	N	H	Sp	Others	Total
1 Original H 26-92/4	1926	D1	1927	3 —	150	121	22	77	0	0	99
2 H ex H	1927	D2	1928	12 —	444	393	59	330	0	0	389
3 Resowing	—	—	1930	— 1	3	3	0	2	0	1 SC	3
4 H ex H	1928	D3	1929	5 —	275	230	49	181	0	1 dwf.	231
5 H ex H	1929	D4	1930	6 —	288	279	45	226	3	1 dwf.	275
6 Total	—	—	—	26 1	1160	1026	175	816	3	3	997
7 N ex H	1927	D2	1928	2 —	24	22	21	1	0	0	22
8 Total N progeny	'27, '29	D2, 3	'28, '30	5 1	169	161	160	1	0	0	161

One of the het speltoids in D<sub>2</sub> was superficially a chimera. It bore three het speltoid heads and one that was normal on one side and het speltoid on the other. The seeds of this head were all numbered according to their position and planted separately following the system of Åkerman (2). There was no correlation between glume type and the genetic constitution of the seeds; the normal side of the head gave the same segregation as the rest of the plant.

The chromosome constitution of the subcompactoid sib was  $19_{II} + 1_I + C_1 + Cil$ , which is the same as that of a Type I subcompactoid except that one unidentified chromosome was lacking.

Six het speltoid plants of this strain were found by Huskins (9) to have  $20_{II} + C_1$ . Four more since studied in smear preparations have the same. In these acetocarmine preparations, however, part of the univalent distal to the secondary constriction often appears to be a little broader at metaphase than in the preceding three strains, see Fig. 5.

Counts of micronuclei were made in 150 het speltoid pollen tetrads. They indicate that 83.5% of the pollen grains contain only 20 chromosomes.



FIG. 5.  $\beta$  4 het speltoid;  $20_{II} + C_1$ .

Two normal segregates examined by Huskins (9) and one examined since had  $21_{II}$ . The three dwarf speltoid segregates were all too mature for cytological study when first identifiable. They were sterile. Three of the  $D_2$  het speltoids were sent to Dr. Uchikawa, who obtained in four years a total of 374 N, 1750 H, 28 Sp, 9 SC, 5 "Short compactum", and 3 "Dwarf compactum" (36). He found  $20_{II}$  in most p.m.c. of the speltoids, but some univalents and multiple associations. They were reported to be about 50% fertile—but see later.

## B. SPELTOIDS OF SERIES $\gamma$

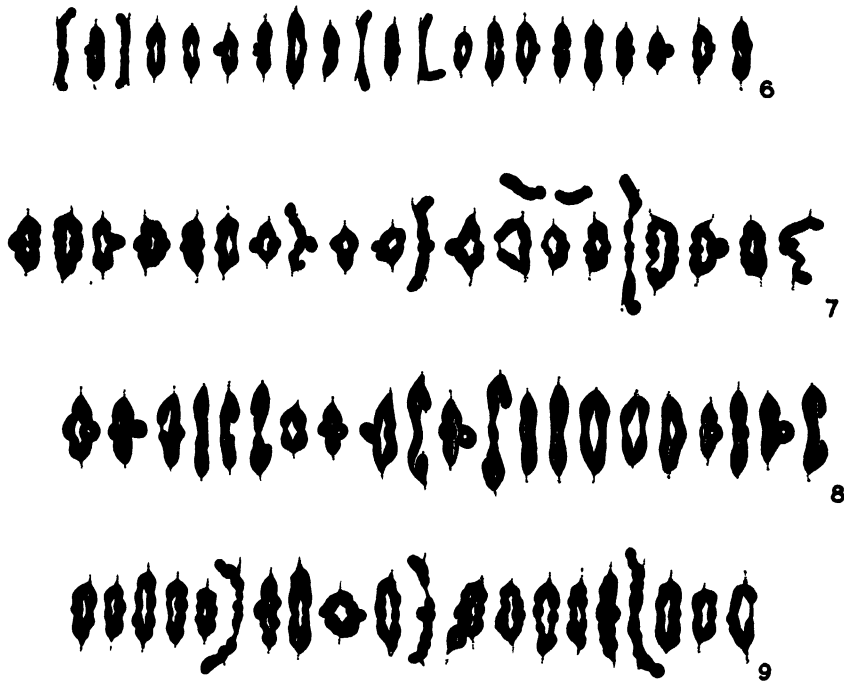
### (a) Strain $\gamma$ 1 (Fig. 29)

This was Åkerman's 1924-444 when two speltoids (26-62/3) and three het speltoids (26-64/6) were obtained from him in 1926. On the basis of Nilsson-Ehle's definition he had originally classified it as Series A rather than C because it did not produce more normals than het speltoids. It has given us (Table VI) 161 N, 207 H, and 13 Sp and we class it as Series  $\gamma$ .

TABLE VI

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny			
		No.	Sown		Sown	Germ	N	H	Sp	Total
1 Original H 26-64/6	1926	D1	1927	3 —	120	92	40	48	2	90
2 H ex H	1927	D2	1928	4 —	132	97	40	54	2	96
3 Resowing	—	—	1930	— 1	4	4	2	2	0	4
4 Resowing	—	—	1935	— 1	48	30	9	16	0	25
5 H ex H	1928	D3	1929	3 —	192	167	65	81	8	154
6 H ex H	1930	—	1936	1 —	36	12	5	6	1	12
7 Total	—	—	—	11 2	532	402	161	207	13	381
8 Original Sp 26-62/63	1926	D1	1927	2 —	60	29	0	1	26	27
9 Total Sp progeny	'26, '27	D1, 2	'27, '28	9 —	203	143	0	1	136	137
10 N ex H	1927	D2	1928	9 —	180	153	152	0	0	152
11 Sp ex H	1927	D2	1928	1 —	24	20	0	0	20	20
12 Total Sp progeny	'27-'30	D2, 3	'28-'36	3 1	61	43	0	1	41	42

Five het speltoids had  $21_{II}$  in most p.m.c. nuclei, but one of these bivalents was heteromorphous (Fig. 8). In a sample of 50 cells the heteromorphous pair were unassociated in six (Fig. 9). The larger member when unassociated is



FIGS. 6 to 9. Segregates of strain  $\gamma$  1:

6. Het speltoid with  $20_{II} + CCd_{II}$ .
7. The same with C and Cd unpaired.
8. Normal with  $21_{II}$ .
9. Speltoid with  $21_{II}$  (two bivalents interlocked).

similar in form to the univalent C chromosome of  $\beta$  het speltoids. The shorter member is only about three-quarters as long and the pairing relationships indicate a deficiency in that portion of the C chromosome major arm distal to the secondary constriction – see description of the heteromorphous pair in Strain  $\gamma$  2 and Figs. 11 and 12 from it. When unassociated, these “C” and “C-deficient” (“Cd”) chromosomes move on to the plate and “split” during the first anaphase (Fig. 36) as does the univalent in Series  $\beta$ . A sixth het speltoid had  $20_{II} + C$  (Series  $\beta$ ). It arose from a het speltoid known to have  $20_{II} + C + Cd$  (Series  $\gamma$ ). Three of its het speltoid sibs had the same constitution as the parent and a normal sib had  $21_{II}$ .

One  $D_4$  het speltoid obviously arose from natural crossing. It was brown and its progeny comprised 12 brown and six white N, 19 brown and seven white H, and two brown Sp. They varied in length of awns. One of these het speltoid progeny had a typical Series  $\gamma$  chromosome complement, but another had a Series  $\beta$  complement.

Three normals, two examined by Huskins (9) and one since, all bred true and have normal chromosome number and behavior (Fig. 8).

The two speltoids obtained from Åkerman and their speltoid progeny gave 136 Sp and 1 H. Three speltoids from het speltoids gave 41 Sp and 1 H progeny. The first het speltoid gave 18 N and 17 H progeny and segregated for glume color; it therefore arose from pollination by a normal gamete from a different strain. It should be stressed that no well-established case (i.e. one in which the possibilities of natural crossing or seed admixture are ruled out) is known of reversion from speltoid to het speltoid or normal.

A speltoid of this strain had 21 homomorphic bivalents (Fig. 9). Presumably one of these is a pair of Cd chromosomes but they cannot be identified when all the bivalents are homomorphic. The shorter length of the Cd is obvious when it is accompanied by a normal C chromosome in the het speltoids, but a CdCd bivalent in a speltoid is no smaller than some of the other bivalents.

Some of the progenies from these speltoids were weak and more or less sterile plants while others were little different from het speltoids of this strain in vigor and fertility. It can never be taken for granted that speltoids from  $\beta$  or  $\gamma$  het speltoids or speltoids are themselves of the parental series, for both natural crossing and secondary chromosome aberrations are common and either may have entered into their origin. In the present case it seems probable that the weaker speltoid progenies were true Series  $\gamma$  and the others not.

#### (b) Strain $\gamma$ 2 (Fig. 31)

This is the strain which Philiptschenko (29) reported as giving a  $\gamma$  progeny ratio while having a  $\beta$  chromosome constitution. The cytological study cited by Philiptschenko was carried out by Vasiliev (37) who found  $21_{II}$  in pollen mother cells of normal plants,  $20_{II} + 1_I$  in het speltoids and 40 chromosomes in root tip cells of a speltoid segregate.

The strain originated from a het speltoid found by Philiptschenko in an  $F_2$  of *T. compactum creticum*  $\times$  *T. vulgare* var. Marquis. In 1927 ( $F_2$  of the hybrid family) the original het speltoid gave him 20 N and 25 H progeny. In 1928 he sowed 450 seeds from six normal plants, but owing to unfavorable weather conditions obtained only 204 mature plants. All were normals. From eight het speltoid plants 620 seeds were sown, but only about 200 progeny grew and many were badly developed. Only 174 could be classified, these consisted of 68 N, 100 H, and six Bearded Sp. Detailed descriptions and measurements of all three types are presented by Philiptschenko and the differential mortality of het speltoid progeny is discussed. For present purposes we need cite only the fact that five speltoid culms were just slightly taller than 80 culms of their het speltoid sibs. Presumably each culm represented a different plant, but this is not specifically stated. In view of our later findings, it is important to note that in any case at least one speltoid segregate was not measured. This may have been the one used for cytological

study and found to have only 40 chromosomes. We have never, either in wheat or oats, found 40-chromosome plants taller than their 41- or 42-chromosome sibs.

In 1929 heads of three het speltoid plants (probably  $F_4$ ) were obtained from Professor Philiptschenko. They were more like *T. spelta* than any of our other speltoid strains excepting those plants of the  $\alpha/\gamma$  strain (Smith, unpublished) which almost surely had a *T. spelta* ancestor. Seeds of the three het speltoids were sown in 1930 under the numbers 29-226/228 (Table VII).

TABLE VII

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny			
		No.	Sown		Sown	Germ.	N	H	Sp	Total
1 Original H 29-226/228	1929	D1*	1930	3 —	78	75	33	41	1	75
2 Resowing of 29-228			1935	— 1	36	25	10	11	0	21
3 $\gamma$ H segregates	1930	D2	1931	5 —	120	95	50	41	3	94
4 Resowings			1932	— 3	72	50	19	30	0	49
5 Resowing			1935	— 1	36	12	7	5	0	12
6 Resowing			1936	— 1	48	11	4	4	0	8
7 $\beta$ H segregates ex $\gamma$			1931	3 —	72	54	7	44	1	52
8 $\gamma$ H segregates	1931	D3	1932	8 —	192	170	73	90	3	166
9 Resowings			1934	— 3	72	53	28	21	1	50
10 $\beta$ H segregates ex $\beta$			1932	3 —	72	61	10	50	1	61
11 $\beta$ H segregates ex $\beta$			1934	1 —	30	26	3	23	0	26
12 $\gamma$ H segregates			1935	3 —	132	107	43	54	2	99
13 Resowing			1937	— 1	48	12	5	7	0	12
14 Total for Series $\gamma$	—	—	—	19 10	834	610	272	304	10	586
15 Total for Series $\beta$ 5	—	—	—	7 —	174	141	20	117	2	139
16 N segregate ex $\gamma$ H	1930	D2	1931	1 —	24	23	23	0	0	23
17 Total N progeny	'30, '31	D2, 3	'31, '32	2 —	48	46	45	0	0	45
18 Sp ex $\gamma$ II	1930	D2	1931	1 —	12	7	0	0	4	4
19 Total Sp progeny	'30-'34	D2-4	'31-'35	4 —	38	24	0	0	19	19

\* The first generation from seeds obtained from Professor Philiptschenko; probably  $F_5$  of his cultures.

The  $D_1$  progenies (though small) indicate that the parents probably belonged to Series  $\gamma$  and cytological analysis supports this view. Two phenotypically similar  $D_1$  het speltoids from plant 29-226 were examined. One was a typical Series  $\gamma$  having 42 chromosomes including a heteromorphic pair; the other was a  $\beta$  type having only 41 chromosomes. In  $D_2$  the first plant gave a  $\gamma$  progeny ratio; the second plant gave a  $\beta$  ratio. Similarly from  $D_1$  family 29-227 one het speltoid was found to have 42 chromosomes including a heteromorphic pair and another had only 41. Again the progeny ratios followed

cytological expectation. From D<sub>1</sub> family 29-228 the one normal plant examined had 21<sub>II</sub>, three het speltoids had 20<sub>II</sub> + CCd, and one het speltoid had 20<sub>II</sub> + C<sub>I</sub>. The normal bred true, the two CCd het speltoids tested gave  $\gamma$  ratios, and the 20<sub>II</sub> + C<sub>I</sub> plant gave a  $\beta$  ratio. One het speltoid from family 29-228 which was not examined cytologically gave a Series  $\gamma$  ratio. The one speltoid segregate tested bred true.

In succeeding generations het speltoids of the  $\beta$  Series (Strain  $\beta$  5) continued to give  $\beta$  ratios, while  $\gamma$  het speltoids continued to give both  $\beta$  and  $\gamma$  het speltoid progeny. Cytological examinations or genetic tests, or both, were made of 36 het speltoid progeny of  $\gamma$  het speltoids. Of these, 31 were  $\gamma$  and five were  $\beta$ .

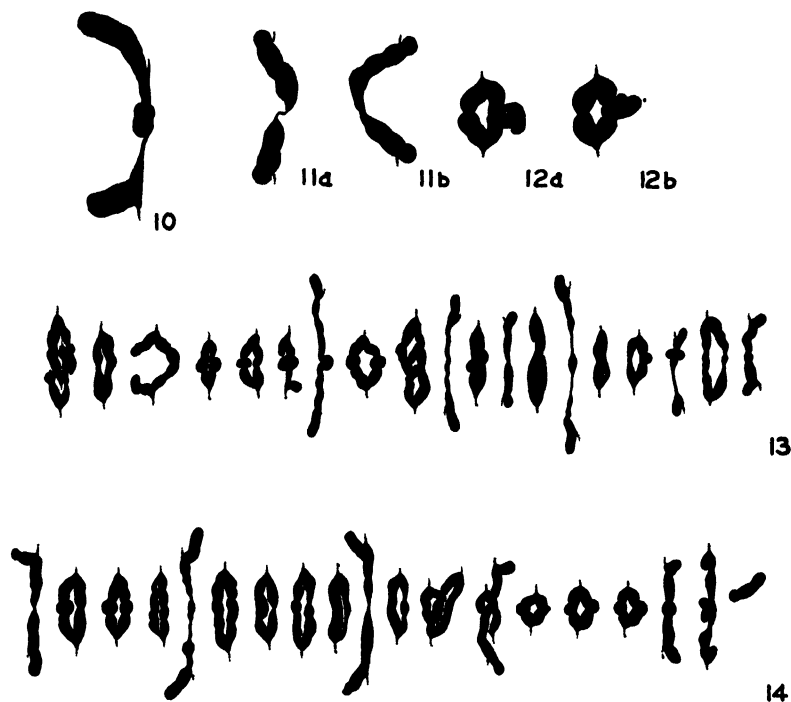
Four normals all had 21 homomorphic pairs. Nine het speltoids which gave  $\beta$  ratios all had 41 chromosomes. Four of them were derived directly from  $\gamma$  het speltoids and five were from  $\beta$  het speltoid segregates. They usually had 20<sub>II</sub> + 1<sub>I</sub> but a few cells were seen with 19<sub>II</sub> + 3<sub>I</sub>. Observations on 150 first division telophases and 150 pollen tetrads indicate that the majority of male gametes formed have only 20 chromosomes. That very few of these function in fertilization is shown by the total segregation of 20 N, 117 H, and only 2 Sp (Table VII).

One  $\beta$  het speltoid segregate had 42 chromosomes, as had also three of its het speltoid descendants, but these never formed 21<sub>II</sub>. Characteristically there were 19<sub>II</sub>, a C<sub>I</sub>, and a non-C trivalent. The non-C trivalent was also found in fertile speltoid segregates (Fig. 40). There is evidently the usual unpaired C chromosome and an extra chromosome other than C involved in this case. This chromosome appears to compensate for the lack of a C in that it apparently increases the functioning capacity of speltoid-determining pollen grains. The ratio from this het speltoid and three similar progeny was 21 N : 111 H : 21 Sp, which is rather like the "modified  $\beta$ " ratio found in fatuoids.

In the  $\gamma$  het speltoids of this strain the CCd chromosomes are rather frequently unassociated at metaphase. In a sample of 154 p.m.c. they were associated in 133 (Fig. 37) and unassociated in 21 (Fig. 35). Very occasionally two pairs were unassociated; it could not be determined whether the second pair was the same in all cases.

It seems evident that, as in the previous strain, the difference in size between the members of the heteromorphic pair of  $\gamma$  het speltoids is due to an interstitial deficiency in one of them. The size difference is considerable (about 26%); therefore were it due to translocation, associations should sometimes be formed with other chromosomes and none was observed. Likewise, were it due to a duplication in the larger member this might sometimes form a loop or ring within itself and this was not seen. The larger member appears identical in form with the univalent of  $\beta$  het speltoids of this and other strains, which together with the genetic data supports the deficiency interpretation.

That it is an interstitial deficiency is indicated by the fact that the pair can be associated terminally by either the short arms (Fig. 10) or the unequal long arms (Figs. 11a and 11b). That it is situated in the distal half of the



FIGS. 10 to 14. Segregates of strain  $\gamma$  2:

10. *C* and *Cd* united by their short arms.
11. *C* and *Cd* united by their long arms—one normal and the other segmentally deficient.
12. *C* and *Cd* united by both arms.
13. Speltoid with  $21_{II}$  (two complete interlocks).
14. Speltoid with  $20_{II} + Cd_I$  (one complete and one incomplete interlock).

major arm is indicated both by its shape and by the fact that where associations occur like those in Figs. 12a and b, in which a chiasma is present about halfway along the longer member, the regions distal to it are of unequal length.

When *C* and *Cd* are unassociated at first metaphase they often fail to become included in the first or second division nuclei. By the recombination of  $20 + Cd$  and 20-chromosome gametes, speltoids of three chromosomal types,  $40 + 2 Cd$ ,  $40 + Cd$ , and 40, will be expected in the progeny of  $\gamma$  het speltoids but the speltoids segregated by  $\beta$  het speltoids should all be of the 40-chromosome type. The 40-chromosome type was found by Vasiliev (37) but it is not certain whether it was the progeny of a  $\beta$  or  $\gamma$  het speltoid. We have obtained the other two types, though from a speltoid segregate of 29-228 and not directly from a het speltoid; they are phenotypically alike. The 42-chromosome speltoid usually had  $21_{II}$  (Fig. 13) but there was a high



frequency of interlocking at metaphase. Two speltoids with 41 chromosomes usually had  $20_{II} + 1_I$  (Fig. 14). The behavior of the univalent, which from its shape seemed to be a Cd chromosome, was like that of the C chromosome in  $\beta$  het speltoids. Speltoids of this type should, therefore, produce two types of gametes, one with  $20 + Cd$ , the other with only 20 chromosomes and, apart from natural crossing, their offspring should all be speltoids, as eight progeny from one such plant were found to be. The speltoids with  $20_{II} + CdCd$  and those with only  $20_{II}$  apparently differ only in vigor and fertility.

The high frequency, 14%, with which  $\beta$  het speltoids arise from  $\gamma$  in this strain was evidently responsible for the difficulty Philipschenko experienced in his analysis. To harmonize his ratio with our joint  $\beta$  and  $\gamma$  ratios it is only necessary to assume that two of the eight het speltoids he tested were  $\beta$  segregates, as was the speltoid in which Vasiliev counted 40 chromosomes.

(c) Strain  $\gamma$  3 (Fig. 28)

During the winter of 1939-40 two "compact ears" of wheat and two "long-eared" were received from Dr. S. J. Wellensiek. The "long-eared" plants of this strain were said to segregate "compact", "long", and "speltoid" in the ratio of approximately 5 : 4 : 1 and the "compact" to breed true. The long-eared type is not unlike the normals of some of our other strains in either glume shape or head laxity, but it is intermediate between the "compact" type and the speltoid. The "compact" type is not unlike some of the sub-compactoid mutations to be dealt with later, but has squarer glumes than any of them. As emphasized in (12), varieties of *T. vulgare* differ greatly with respect to the characteristics involved in the "speltoid complex" or its "normal" counterpart and the classification of the normal and het speltoid segregates from any given het speltoid can only be relative to the characteristics of the variety from which it arose. These "compact" and "long" ears are the "normal" and "het speltoid" type for this strain, though the latter has little resemblance either to ordinary speltoids or to *T. spelta*.

Three seeds from each normal ("compact") head and 12 from each het speltoid ("long") were sown immediately in the greenhouse. The normals gave normals and the het speltoids gave 6 N, 12 H, and 1 Sp. The speltoid was typical in having lax heads with full beards and sharply keeled, indurated glumes. It was taller than its normal and het speltoid sibs.

Two normals had  $21_{II}$ . The het speltoids had either  $20_{II}$  plus one heteromorphic pair (Fig. 15) or  $20_{II}$  plus two univalents of different length.



FIG. 15.  $\gamma$  3 het speltoid;  $20_{II} + CCd_{II}$ .

When unpaired the two looked like the C and Cd chromosomes of other Series  $\gamma$  strains. A  $D_2$  sowing made in the spring of 1942 to determine the genetic ratio failed to head; it is evidently a winter wheat.

(d) Strain  $\gamma$  4 (Fig. 30)

Three plants classified under his numbers 26-1295<sub>1-3</sub> as het speltoids of Series C were obtained in 1927 from Professor Nilsson-Ehle. Their progeny are recorded in Table VIII where it will be seen that the strain now comprises three types treated here as substrains, viz., (a) the het speltoids which continued segregating as Series  $\gamma$ ; (b) het speltoids segregating as Series  $\alpha$ ; and (c) a het speltoid with extremely narrow glumes which segregated normals, het speltoids like itself, and speltoids, also with atypical narrow glumes; this form arose among the progeny of a normal segregate of substrain (a). In addition, the expected Series  $\beta$  arose from two  $D_2$  het speltoids of substrain (a) which were given to Dr. I. Uchikawa (36).

Substrain (a)

This strain gave 230 N : 271 H : 1 Sp. Uchikawa (36) obtained from it 347 N, 380 H, and 21 Sp. This is a  $\gamma$  strain on our definition; though obtained directly from Professor Nilsson-Ehle as Series C it has not proved to be such on his definition which requires an excess of normals.

The segregated normals and their normal offspring gave 452 normals, three ordinary het speltoids, and the narrow-glumed het speltoid progenitor of substrain (c). One of the "normals" which gave a het speltoid had only 41 chromosomes (9) and the parents of the other two were dwarf plants and may therefore also have been chromosomally unbalanced. One of the het speltoids was sterile. One had brown glumes and was clearly a hybrid with another strain. That which arose from the 41-chromosome "normal" resembled other het speltoids of this strain. It gave 17 N and 13 H progeny.

Six normals of substrain (a) had  $21_{II}$ . A seventh had  $21_{II}$  plus a fragment, Fig. 16. The fragment was not always detectable at metaphase, probably because it was sometimes paired with one of the bivalents. This plant bred true for the "normal" phenotype and the only one of its progeny examined had  $21_{II}$  and a fragment like that of its parent. The 41-chromosome "normal" which gave rise to the het speltoid gave in addition 64 normal progeny. Two of these were examined and each had  $21_{II}$ . The chromosome missing from the parent plant was evidently not a C chromosome.

Huskins (9) reported that the het speltoids of this substrain had 43 chromosomes "probably usually arranged as  $20_{II} + 1_{III}$ ". In one, however, he found "a further cytological abnormality in the loss of approximately half a chromosome." This was later, however, found to be the primary characteristic of these het speltoids and we now regularly find  $21_{II}$  (Fig. 17) of which one is extremely heteromorphic. The smaller member is about as long as the short

TABLE VIII

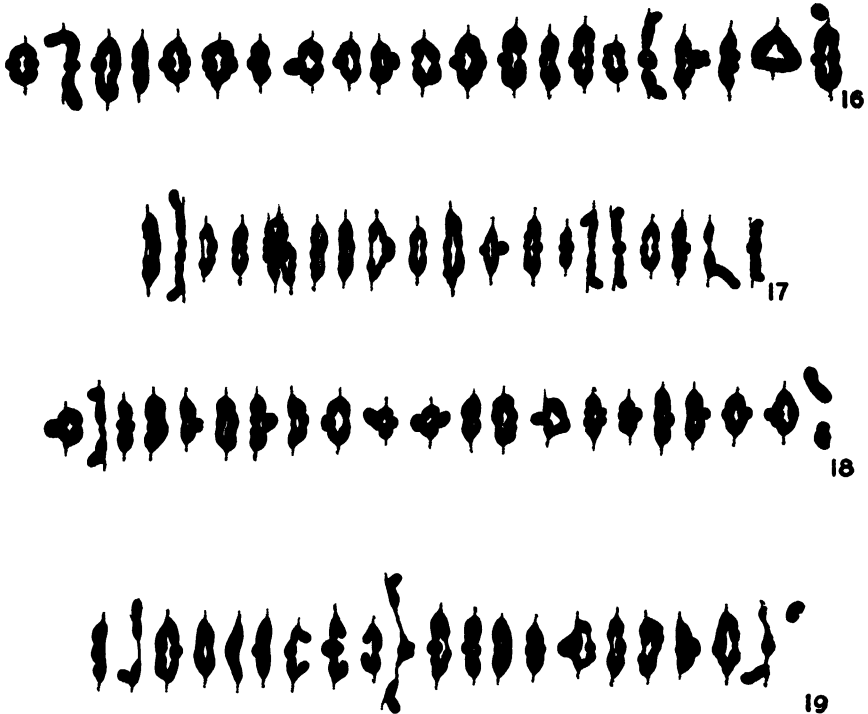
Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny				
		No.	Sown		Sown	Germ.	N	H	Sp	Others	Total
(a) 1 Original H 26-95/97	1926	D1	1927	3 —	150	116	48	67	0	0	115
2 Resowing 26-96	—	—	1935	— 1	24	11	7	1	0	0	8
3 H ex H	1927	D2	1928	5 —	240	193	77	95	0	0	172
4 H ex H	1935	—	1936	1 —	17	4	1	3	0	0	4
5 H ex H	1928	D3	1929	6 —	252	207	97	105	1	1*	204
6 Total	—	—	—	15 1	683	531	230	271	1	1*	503
7 N ex H 26-97	1927	D2	1928	1 —	36	34	34	0	0	0	34
8 N ex H 26-96	1935	—	1936	1 —	24	11	10	0	0	0	10
9 41-chr. N ex H 26-95	1927	—	1928	1 —	72	65	64	1	0	0	65
10 N ex H	1928	D3	1929	3 —	102	93	91	0	0	1**	92
11 N ex 41-chr. N	—	—	—	3 —	70	63	36	0	0	0	36
12 Dwf. N ex Dwf. N	1929	D4	1930	4 —	108	105	103	2	0	0	105
13 Dwf. N ex Dwf. N	1930	D5	1931	2 —	48	46	43	0	0	0	43
14 Superficial chimera	—	—	—	1 —	88	71	54	0	0	0	54
15 Dwf. N ex Dwf. N	1931	D6	1932	2 —	24	19	17	0	0	0	17
16 Total	—	—	—	18 —	572	507	452	3	0	1**	456
17 H ex 41-chr. N	1928	D3	1929	1 —	36	31	17	13	0	0	30
(b) 18 Hybrid H ex 26-96	1927	D2	1928	1 —	36	31	8	14	7	0	29
19 H ex hybrid H	1928	D3	1935	2 —	48	35	12	8	4	0	24
20 Resowing	—	—	1936	— 1	48	6	3	1	0	0	4
21 H ex H	1935	D4	1940	3 —	96	54	16	21	14	1†	52
22 Total	—	—	—	6 1	228	126	39	44	25	1	109
23 Sp ex hybrid H	1928	D3	1929	2 —	48	40	0	0	40	0	40
(c) 24 "H" ex N	1929	D4	1930	1 —	24	22	10	12	0	0	22
25 "H" ex "H"	1930	D5	1931	1 —	24	23	11	5	1	0	17
26 Resowing	—	—	1932	— 1	24	22	7	13	0	0	20
27 "H" ex "H"	1931	D6	1932	2 —	48	43	14	26	0	0	40
28 Total	—	—	—	4 1	120	110	42	56	1	0	99
29 Sp ex "H"	1931	D6	1932	1 —	17	12	0	0	12	0	12
30 Total Sp progeny	'31, '32	D6, 7	'32, '36	2 —	29	17	0	0	17	0	17

\* A natural hybrid which gave 54 N : 17 H and a superficial N-H chimera, which gave only normal progeny, see line 14.

\*\* = "H", a het speltoid with shoulderless and narrow, but typically indurated glumes, see l. 24.

† Dwarf.

arm of a normal C chromosome and is telokinetic; we designate it Cts. It is probable that the three segments of the heteromorphic bivalent were taken to be whole chromosomes in the original counts of 43. The initial correct interpre-



FIGS. 16 to 19. Segregates of strain  $\gamma$  4:

16. "Normal" with  $21_{II} + 1$  fragment.
17.  $\gamma$  het speltoid with  $20_{II} + CCts_{II}$  (three bivalents interlocked).
18. "Normal" from narrow-glumed het speltoid;  $20_{II}$  plus heteromorphic pair, here unassociated.
19. Narrow-glumed het speltoid;  $19_{II} + CCts_{II} + 1_{I}$ .

tation of loss was made from cells in which C and Cts were unpaired. We have now found them unpaired in six out of a sample of 100 cells. Uchikawa (36) also found  $20_{II}$  plus an extremely heteromorphic pair in het speltoid progeny of the plants he received from us. However, in the 3617 cells he examined, he found only about 0.4% with the heteromorphics unpaired. The difference may be due to environmental factors affecting meiosis or may in part be due to the fact that his material, like that of Huskins' original study, was sectioned.

One het speltoid had 19 apparently normal bivalents, the heteromorphic CCts, and a pair of fragments; it bore 32 seeds but from these only three mature progeny, all normals, were obtained.

Only one speltoid arose in this substrain. It was fully bearded and had only one culm. Uchikawa found the expected  $20_{II}$  plus a very small pair, apparently CtsCts, in his speltoids of this strain.

## Substrain (b)

In the  $D_1$  generation of substrain (a), one short-bearded het speltoid arose which gave 8 short-bearded N, 14 short-bearded H, and seven fully bearded Sp progeny (Table VIII). The segregation from later generations was 31 N, 30 H, 18 Sp, and 1 dwarf plant which failed to head and hence could not be classified. The grouped ratio, 39 N, 44 H, and 25 Sp is not significantly different from that of some  $\alpha$  strains. It is quite different from that of the parent strain. It appears that this substrain arose from crossing, while still in Nilsson-Ehle's cultures, with either *T. spelta*, or more probably, with a descendant of crosses between *T. vulgare* and *T. spelta* made earlier by Nilsson-Leissner (26) at Svalöf—the same hybrid material that contaminated our strain  $\beta$  1 before it was obtained from Dr. Åkerman.

Two "het spelta-speltoids" from the  $D_1$  of this hybrid had  $21_{II}$  of which one appeared slightly heteromorphic. The glume type ranged from typical speltoid to typical *T. spelta*, indicating the existence of modifiers segregating independently of the C chromosome in this hybrid. These could, of course, be (or include) alleles (in the phylogenetic sense) of the spelta-speltoid gene series situated on homoeologous chromosomes (B or A of Winge). The heteromorphic pair of this substrain may have originated as a C chromosome from *T. vulgare* and a  $C^s$  chromosome from *T. spelta*. This would account both for the phenotype and the relative fertility of both types of pollen.

Meiotic irregularities similar to those found by Huskins (9) in the spelta hybrid of strain  $\beta$  1 were found in all three of the main types of segregates. Three normals had  $21_{II}$ ; one had 20 homomorphic bivalents and a pair which appeared to be slightly heteromorphic; another had 20 normal bivalents plus a telokinetic pair; a sixth had  $20_{II}$  and a small univalent. Six het speltoids all had one slightly heteromorphic bivalent. Accompanying it, one had the usual  $20_{II}$ ; one had  $19_{II}$  plus two small chromosomes usually unpaired but possibly associated in four of the 109 nuclei examined; another had  $19_{II}$  plus a second heteromorphic pair of which the shorter member was telokinetic; two had  $19_{II} + 1_{III}$ ; another had  $19_{II}$ ,  $1_{III}$ , and a pair of fragments. The only "speltoid" segregate examined had 21 homomorphic bivalents.

## Substrain (c)

A plant with extremely narrow and shoulderless, though typically indurated and keeled, glumes arose in the  $D_3$  generation of substrain (a) together with 27 normals as the progeny of a normal descendant of a het speltoid. Despite its atypical glumes it must be classified as a  $\gamma$  het speltoid (Table VIII). In later generations there was much sterility and many plants had badly formed heads which could not be classified. The genetic record is given only for the early generations. The normals examined had either  $21_{II}$  or  $20_{II}$  plus a heteromorphic pair (Fig. 18). Four constitutions were found in het speltoids:  $20_{II} + CCts$ ;  $19_{II} + CCts$  plus one slightly heteromorphic pair;  $19_{II} + CCts + 1_I$  and  $19_{II} + CCts$  plus one pair of fragments. Apparently the primary

segregation was due to the CCTs pair of the parent stock. The single speltoid examined had  $21_{II}$  with much interlocking.

### C. SPELTOIDS OF SERIES $\alpha$

#### (a) Strain $\alpha$ 1 (Fig. 32)

This strain is derived from a single het speltoid obtained from Dr. Åkerman. It arose in 1926 in the  $F_0$  of a cross Extra-Kolben  $\times$  Schlanstedt. Its progeny ratio, 55 : 95 : 32 (Table IX), is typical for Series  $\alpha$ . Two speltoid segregates gave 41 speltoids and one het speltoid which was a natural hybrid.

TABLE IX

Parent plant	Year grown	Generation		No. of families	No. of seeds		Progeny			
		No.	Sown		Sown	Germ.	N	H	Sp	Total
1 Original H 26-74	1926	D1	1927	1 —	50	44	14	21	9	44
2 H ex H	1927	D2	1928	2 —	72	64	20	34	10	64
3 Resowing	—	—	1930	— 1	3	3	2	1	0	3
4 Resowing	—	—	1934	— 1	12	8	0	8	0	8
5 H ex H	1930	D3	1934	1 —	12	12	3	6	3	12
6 H ex H	—	—	1936	1 —	12	10	3	5	2	10
7 H ex H	1934	—	1935	1 —	48	39	7	16	7	30
8 H ex H	1936	D4	1937	1 —	12	6	3	2	1	6
9 Resowing	—	—	1938	— 1	24	10	3	2	0	5
10 Total of H	—	—	—	7 3	245	196	55	95	32	182
11 N ex H	1927	D2	1928	1 —	12	12	12	0	0	12
12 Sp ex H	1927	D2	1928	1 —	24	22	0	1	21	22
13 Total Sp progeny	'27, '30	D2, 3	'28-'36	2 2	49	42	0	1	41	42

Huskins (9) examined one plant from each class of segregates and reported 42 chromosomes in each. In the normal he found  $21_{II}$ . Multivalents were recorded in the het speltoid and speltoid. There is no doubt that the quadri-valent shown in his Fig. 4 and the univalent in Fig. 2 are correctly interpreted, but Figs. 1, 2, and 3 are now considered doubtful interpretations. In smears we have found interlocked bivalents but no multivalents. Uchikawa (36) found quadrivalents in about 0.3% of the nuclei of all three classes of segregates, but there is a possibility that his material had a hybrid origin (mith, unpublished). It is now clear from Uchikawa's and our own work that trivalent formation is not a special characteristic of  $\alpha$  het speltoids, nor quadrivalent formation of its speltoids. Typical metaphase plates of these het speltoids and speltoids are shown in Figs. 20 and 21.

The bivalents always appear to be composed of homomorphic pairs in the normals and speltoids and usually in the het speltoids also. When a bivalent associated at only one end is present in the het speltoids it sometimes appears



FIGS. 20, 21. Segregates of strain  $\alpha$  1:

20. Het speltoid with  $21_{II}$ .

21. Speltoid segregate with  $21_{II}$  (two complete interlocks).

to comprise a heteromorphic pair. The degree of inequality, however, is so slight that it is difficult to be certain that it is not due to unequal stretching or contraction. As a possible, indirect, test for heteromorphism due to deficiency, pollen tetrads of normals and het speltoids of this strain were compared for the presence of micronuclei. There were 19 in 1727 tetrads from normals and four in 272 from het speltoids. This is 1.10 and 1.46%, respectively and the difference is not significant. If there is a deficiency or any other structural change in one of the C chromosomes of these  $\alpha$  het speltoids it evidently does not affect synapsis of the normal and mutated C chromosomes sufficiently to upset their association and disjunction and thereby increase the frequency of micronuclei.

(b) Strain  $\alpha$  2 (Fig. 33)

This is a "part-mutation" beardless speltoid strain from the variety Iron (*T. vulgare* var. *lulescens*). Two beardless speltoid and two beardless het speltoid plants were obtained from Prof. H. Nilsson-Ehle and mature progeny were obtained from seed sown in England in March 1930. None of their progeny headed when sown in Canada the following spring. The two speltoids bred true. The two het speltoids gave 17 N, 25 H, 22 Sp, and 1 normal-het speltoid chimera, all beardless. The deviation from a 1 : 2 : 1 ratio though considerable is not significant ( $\chi^2 = 3.84$ ;  $P = .10 - .20$ ).

Two normal plants regularly had  $21_{II}$ ; in only one of the large number of nuclei examined were two chromosomes unpaired. Four het speltoids all had  $21_{II}$  including one that sometimes appeared to be very slightly heteromorphic (Fig. 22) and failure of association of one pair was noted in a few cells. Three speltoid segregates all had  $21_{II}$ .

The chimera had one het speltoid and two normal heads. By a fortunate chance, one culm had been fixed—of course before the phenotype was determinable. It had one heteromorphic bivalent (Fig. 41), an arm of one chromosome having been lost. Genetic analysis would, of course, be necessary to prove that it is this deficiency which has given rise to the het speltoid phenotype. When the plants produced from seeds of this chimera failed to head in 1931 they were transplanted into the greenhouse but only one



FIG. 22.  $\alpha$  2 het speltoid with 20<sub>II</sub> plus one possibly heteromorphic bivalent—second from right.

survived to head the following July and it was a normal. In 1932 vernalization of the remaining seeds was attempted, but the treatment given failed to cause heading and none of the plants survived the following winter in the greenhouse. It was noted that progeny of the het speltoid head were weaker than those from the normal head. The coincident appearance of the het speltoid spike and the occurrence of a deficiency similar to that of some  $\gamma$  het speltoids, together with the reduced vigor of the progeny, render it probable that we have here direct evidence on the origin of one het speltoid—through segmental chromosome deficiency occurring in somatic tissue; it is most unfortunate that the genetic evidence needed to complete the case was unobtainable.

#### IV. Interpretation of the Segregation Ratios

##### A. General Genetic Data

Nilsson-Ehle (24) showed that while the deviations of Series A ratios from a simple 1 : 2 : 1 could readily be accounted for by "differential gametic elimination", some more complex explanation was necessary for Series B and C ratios. He applied the concept of heterogamy (then current as a corollary of the "Reduplication" theory of linkage) and contrived a formal explanation which he proposed to test experimentally. In 1927, in his paper on part-mutations (25), he mentioned that both his own and Lindhard's data had shown such an explanation to be inadequate. By that time, of course, the reduplication theory itself was discarded and the chromosome theory of linkage thoroughly established. Winge's chromosome studies of speltoids had appeared and Nilsson-Ehle recognized that they shed new light on the problem but he also pointed out serious limitations in Winge's hypothesis.

Lindhard (16) considered that differential zygotic elimination also was involved in determining the ratios. Later (18) he attempted also an explanation involving a pair of factors L and l which were assumed to influence both the compactness of the spike and the segregation ratios.

Leaving aside for the moment the cytological data now available, the facts which must be considered in interpreting the ratios are as follows:



Series A or  $\alpha$  ratios deviate from the 1 : 2 : 1, expected from random segregation and recombination of a unitary difference, only in having an increased proportion of normals, usually at the expense of homozygous mutant progeny. Either (a) "certation", i.e., reduced functioning of mutant gametes when in competition with normal ones, or (b) differential zygotic elimination, i.e., lowered viability of het speltoid and even more so of speltoid zygotes, would suffice to account for the deviations.

Series B or  $\beta$  ratios must depend upon some mechanism which assures the production of a large preponderance of mutant ovules while mutant pollen must either be almost entirely lacking, or else mostly nonfunctional. The converse possibility of functional mutant pollen and nonfunctional mutant ovules (which would, of course, fit self-fertilized het speltoid ratios) is ruled out by the results of reciprocal crosses: het speltoid ♀  $\times$  normal ♂ gives ratios similar to self-fertilization of het speltoids while the cross normal ♀  $\times$  het speltoid ♂ gives almost entirely normal offspring—see later.

Series C ratios as defined by Nilsson-Ehle comprise slightly more normal than het speltoid progeny while speltoid segregates are rare. Evidently, Series C mutant pollen functions less often than in typical Series  $\alpha$ , while the excess of normals demands either, (a) that mutant ovules function, and probably are formed, less often than in Series  $\alpha$ , or else, (b) that there is a greater zygotic elimination of het speltoids in Series C. In the strains we classify as Series  $\gamma$  there is no excess of normal over het speltoid segregates. There is therefore in them no need to assume the production or functioning of more normal than mutant ovules as Nilsson-Ehle did for Series C. It seems possible that lowered viability of het speltoid zygotes during the winter, together with chance variation, may be responsible for the excess of normals in some of Nilsson-Ehle's strains. Until this is ruled out it is unnecessary to speculate further, though it is theoretically possible that het speltoids could sometimes arise through trisomy involving "primary" speltoid genes. If such occur, they could readily produce more normal than mutant ovules, as do some subcompactoids. There is no clear dividing line between  $\gamma$  and  $\alpha$  ratios and the only definite genetic reason for differentiating them is that Series  $\beta$  het speltoids continuously arise from  $\gamma$  and scarcely ever from typical  $\alpha$ . Their continuous, though irregular, occurrence indicates that some abnormal segregation process occurs during ovule formation in  $\gamma$  het speltoids.

### *B. General Cytological Data*

The cytological studies add the following data:

In Series  $\alpha$  the normal chromosome number is present in all three classes of segregates and there is little evidence of any grossly abnormal chromosome behavior during meiosis. The alternative explanations that have to be considered to account for the origin and segregation ratios of  $\alpha$  het speltoids are (a) gene mutation and (b) chromosome aberration, most probably of the deficiency type.

In Series  $\beta$  the presence of an unpaired C chromosome, one or both chromatids of which very frequently fail to become included in the spore nuclei, provides the mechanism for the production of a great excess of mutant (20-chromosome) gametes in both pollen and ovules.

In Series  $\gamma$  there is in one member of a pair of heteromorphic chromosomes a deficiency that both affects the regularity of their meiotic metaphase association and permits the expression of the speltoid characters. When these heteromorphic chromosomes (CCd in some strains and CCts in others) are associated and disjoin normally, mutant and normal gametes are formed in equal numbers. When they are unassociated, both behave as univalents and, as in Series  $\beta$ , some or all of the chromatids may be left out of the spore nuclei. Gametes with only 20 chromosomes are therefore formed fairly frequently and an ovule possessing neither C nor Cd or Cts when fertilized by normal pollen produces a Series  $\beta$  het speltoid.

### C. Ovule Competition and Embryonic Viability

While the above features establish the major differences between  $\alpha$ ,  $\beta$ , and  $\gamma$  Series, we have to consider further various factors which may modify the ratios characteristic of each. In all three Series, reciprocal crosses show that mutant pollen is at a disadvantage when in competition with normal, the degree increasing in the order  $\alpha$ ,  $\gamma$ ,  $\beta$  (see section F, Table XII).

The question remains, are mutant ovules at any disadvantage? Kihara (14) attempted to explain the segregation of pentaploid wheat hybrids in part by the assumption of either ovule sterility, or zygotic elimination during the embryonic stage and his students up to and including Nishiyama (28) and Uchikawa (36) have continued to maintain this for both fatuoids and speltoids, as well as interspecific hybrids. Watkins (38, 39), however, showed clearly that under the conditions at Cambridge ovules with chromosome numbers ranging from 14 to 21 almost all function and produce viable seed. In view of this, it would be surprising if mutant ovules, or embryos, of any of the speltoid Series were seriously handicapped, except perhaps under very adverse conditions and strong competition. Lindhard (16) found in Denmark that het speltoids of Series  $\beta$  were only 64% fertile, while their normal sibs were 84% fertile and he used these data in his interpretation of the ratios, assuming that het speltoid embryos were less, and speltoid embryos much less, viable than normals. Uchikawa (36) has reported that in Japan Series  $\alpha$  het speltoids are only about 1% less fertile than their normal sibs, but that fertility in Series  $\gamma$  is 6.5% and in Series  $\beta$  12.56% lower. On the other hand, Nilsson-Ehle (24) found in Sweden no appreciable difference: In Series  $\gamma$  the percentages of sterile flowers were 6.3 and 7.4 and in Series  $\beta$  they were 9.7 and 9.4 in normals and het speltoids respectively.

These apparently contradictory data can actually be harmonized without taking into account possible differences in the growing conditions. First,

Nilsson-Ehle (24) counted seed production only in the first two flowers of each spikelet, whereas Lindhard counted all florets. Watkins (38) pointed out that: "Whether or not a grain is set in the third, fourth, fifth, or higher flowers of a spikelet depends more on the vigour of the tiller bearing the ear in question than on the amount of sterility; in small ears it is rare to find flowers above the third giving a grain while in ears that are well developed even the sixth flower may sometimes do so. Mean number of grains per spikelet is therefore too much influenced by conditions other than amount of sterility to be a measure of this latter quantity." It was found that the total seed production per open-pollinated spike is similar in normals and het speltoids of both Series  $\beta$  and  $\gamma$  when grown under favorable conditions in England (Table X). In counts restricted to the most favorably situated florets, that is, to the first two florets of spikelets situated between the basal spikelet and the apical three, there was, surprisingly, a smaller actual proportion of sterile florets in the het speltoids than in their normal sibs (Table XI), but in no case was the difference statistically significant. A third test was made by removing, soon after heading, the basal and three apical spikelets completely and, from the remaining spikelets, all florets above the first two, which is the procedure usually followed when crosses are to be made. These heads were left uncovered and free to effect self-fertilization, or to be cross-fertilized occasionally, as in the controls. The progeny ratios obtained from their seeds were in no case different from seeds of untreated heads. In Strain  $\beta$  1 the seeds from treated heads gave 9 N : 39 H : 2 Sp while the control gave 8 N : 42 Het : 0 Sp. In Strain  $\beta$  2 the ratios were 12 : 35 : 0 and 19 : 74 : 0. In Strain  $\gamma$  1 they were 31 : 42 : 5 and 34 : 39 : 2 and in  $\gamma$  4 they were 41 : 45 : 1 and 37 : 48 : 0. The average seed weight from treated and untreated heads was also similar. There were, however, more cross-fertilized seeds on the treated heads; their progenies were excluded from the above ratios.

TABLE X

THE NUMBER OF SEEDS PER SPIKE IN NORMAL AND HET SPELTOID SIBS\*

Strain	Normal			Het speltoid			Difference in number of seeds
	No. of plants	No. of spikelets per head	Mean number of seeds	No. of plants	No. of spikelets per head	Mean number of seeds	
$\beta$ 1	13	20.8 $\pm$ 0.2	37.8 $\pm$ 2.6	13	20.2 $\pm$ 0.3	42.9 $\pm$ 1.8	5.1 $\pm$ 3.1
$\beta$ 3	22	25.3 $\pm$ 0.3	—	50	24.4 $\pm$ 0.2	—	—
$\beta$ 4	17	21.2 $\pm$ 0.3	35.7 $\pm$ 2.2	17	21.7 $\pm$ 0.3	38.2 $\pm$ 2.6	2.5 $\pm$ 3.4
$\gamma$ 1	25	19.9 $\pm$ 0.4	39.2 $\pm$ 2.5	22	19.5 $\pm$ 0.4	38.6 $\pm$ 2.0	0.6 $\pm$ 3.1
$\gamma$ 4	18	21.7 $\pm$ 0.3	40.5 $\pm$ 2.1	18	20.9 $\pm$ 0.3	44.3 $\pm$ 2.6	3.8 $\pm$ 3.3

\* Counted on best spike from each plant. Plants grown under very favorable conditions at John Innes Institution, Merton, in 1929.

TABLE XI

STERILITY OF MOST FAVORABLY SITUATED FLORETS IN NORMAL AND HET SPELTROID SIBS\*

Strain	Normal		Het speltoid		Significance of difference
	No. of plants	Percentage of sterile 1st or 2nd florets	No. of plants	Percentage of sterile 1st or 2nd florets	
$\beta$ 1	13	8.23 $\pm$ 2.67	13	4.04 $\pm$ 2.56	4.19 $\pm$ 3.68
$\beta$ 3	22	25.68 $\pm$ 3.05	50	16.09 $\pm$ 2.15	9.59 $\pm$ 3.73
$\beta$ 4	17	9.61 $\pm$ 3.14	17	6.76 $\pm$ 2.35	2.85 $\pm$ 3.92
$\gamma$ 1	25	10.26 $\pm$ 3.68	22	4.32 $\pm$ 1.26	5.94 $\pm$ 3.88
$\gamma$ 4	18	6.21 $\pm$ 1.80	18	2.14 $\pm$ 2.30	4.07 $\pm$ 2.91

\* Calculated from counts made on one side of best spike from each plant.

Uchikawa's (36) finding of appreciably greater sterility in  $\gamma$  and still more in  $\beta$  het speltoids than in their normal sibs demands further consideration for he determined the percentage of seed setting only in the first and second florets of each spikelet. The clue to the discrepancy between his results and those of Nilsson-Ehle, Watkins, and ourselves seems to lie in the fact that before flowering time he covered with parchment bags the spikes which were to be used for the later examination of mature grains. This is a very necessary procedure if all-natural crossing is to be avoided, but it introduces a complication which he failed to take into account. Watkins (38) found 13% fewer seeds set on bagged, but otherwise untreated,  $F_1$  pentaploid hybrid heads and 23% fewer on heads with top and bottom spikelets and all upper florets removed, than on untouched heads allowed to self-fertilize naturally. He concluded that where the amount of germinable pollen is small, as in these hybrids, "a comparatively slight change in external conditions may turn the scale from failure to success". The same reasoning, *mutatis mutandis*, will account for Uchikawa's results, including the greater sterility in  $\beta$  than in  $\gamma$  het speltoids under similar conditions: in both it is chiefly normal pollen that functions and in  $\gamma$  het speltoids approximately half the pollen is normal, while in  $\beta$  het speltoids not more than one-fifth of it has the normal chromosome constitution.

It is thus not established that het speltoids show a greater degree of sterility than their normal sibs and there is, therefore, no indication that mutant ovules are at a disadvantage, or that het speltoid, or speltoid embryos, are less likely to begin development than are normal ones. There remains the possibility that they may develop less satisfactorily and that seeds carrying het speltoid, or speltoid embryos, may therefore have poorer germination or poorer seedling growth.

Uchikawa's finding of 50 and 55% fertile seeds on  $\beta$  and  $\gamma$  speltoids is surprising, especially in the former. Our  $\beta$  speltoids are sterile unless they have one or more non-C chromosomes in excess that offset the absence of both C chromosomes (see  $\beta$  3), and Sears (32) reports that his 40-chromosome,

speltoids (nullisomic IX) are male sterile. Further (on the basis of assumptions with which, incidentally, we disagree) Uchikawa's own calculations (36, p. 56-7) on  $\beta$  ratios imply that only about 6% of the speltoid embryos formed by het speltoids develop into fully formed grains; it would therefore be surprising if similar embryos formed on speltoids, which are typically weak plants, should be so much more viable. Further, if Uchikawa's  $\beta$  speltoids were even only partially male sterile, their progeny by open pollination would be expected to comprise many het speltoids. He does not present progeny tests, so that one cannot check this, but lacking evidence to the contrary it is reasonable to assume either (a) that the embryos he counted were predominantly het speltoids (resulting from natural crossing) or (b) that he was dealing, not with typical 40-chromosome  $\beta$  speltoids, but with some of those commonly occurring types that have extra non-C chromosomes.

#### D. Later Zygotic Elimination

Several workers with cereals have assumed differential zygotic elimination some time between germination and maturity to account in part for aberrant progeny ratios and particularly for variations between the ratios obtained from apparently similar heterozygotes in different seasons. Lindhard (16) maintained that such zygotic elimination may have been responsible for his obtaining in 1916 only three or four times as many het speltoids as normals in his  $\beta$  strain that characteristically gave 1 N : 8 Het. His strain was fall-sown and in some seasons as few as 10% of the plants survived the winter. In 1918, 81.25% of the seeds from normal segregates produced mature progeny, compared with 66.3% of those from sib het speltoids. From these data, he assumes a 1 : 5 ratio should really be 1 : 7 and so on. Unfortunately, he does not present in his tables the number of seeds sown and germinated, but only the numbers of plants of the various types harvested. It is therefore impossible to check his hypothesis. Further, many of the ratios he considers distinct are really not heterogeneous by modern  $\chi^2$  tests. Detailed germination results are not given by Philiptschenko (29), but he recorded that only 32% of the progeny of het speltoids survived and that only 28% were fully developed plants, while there was 45% survival in the progeny of normal sibs. From this and the ratio he calculated that het speltoids have only 70-80% and speltoids only 8.5% the viability of normals, and attempted to explain the deviation of his  $\gamma$  ratio from 1 : 2 : 1 on the basis of differential mortality of the three classes of segregates.

The only tabulated germination data seem to be those of Uchikawa (36) who unfortunately combines four years' results, as he also combines  $\beta$  segregation ratios, though he presents annual ratios for Series  $\alpha$  and  $\gamma$ . His results will be considered in conjunction with our own germination data and the finding of Åkerman (1) that the weights of 1000 seeds from normals, het speltoids, and speltoids of a strain classed as Series A (but which we should call  $\gamma$ ) were  $41.4 \pm 0.31$ ,  $36.0 \pm 0.30$ , and  $30.5 \pm 0.42$  gm. respectively. These

weight differences might easily be correlated with differential germination, or they might determine differential elimination of seedlings before emergence above ground.

We have found that the percentage of emergence above ground of seeds from normals is usually greater than that of seeds from het speltoids. However, statistical analysis of  $\beta$  emergence data showed that sowings from normals were relatively uniform, while those from  $\beta$  het speltoids had highly significant heterogeneity mainly associated with seasonal differences, though there are also significant but lesser differences between strains and between different progenies of one strain within seasons. It seems probable that the great heterogeneity of the tests on seeds from het speltoids is due to the low germination percentage of some progenies of plants which bore few seeds. Since larger progenies are necessary to test the ratio type of a het speltoid than to determine the homozygosity of a phenotypic normal, there is always the probability that the sample of seed picked from the former will comprise some smaller or more shrunken seeds, particularly if some of the plants are not prolific. Uchikawa (36) showed lower percentage germination for seeds from het speltoids than normals in each of his four  $\beta$  strains but the difference was not significant in any one strain. In any event, his percentages, being based on four years' lumped data, are conclusive only if their components were not heterogeneous.

Uchikawa's sowings of seeds from  $\beta$  or  $\gamma$  speltoids showed an appreciably lower percentage emergence than those from het speltoids or normals, from which he concludes there is differential elimination in early stages of speltoid zygotes and that this helps to explain the  $\beta$  and  $\gamma$  ratios. However, his difference in emergence is based on much smaller sowings of seeds from speltoids than from het speltoids or normals, and as these smaller sowings are not in rounded or regular numbers, the probability arises that, as in our own het speltoid sowings, there may have been included an undue proportion of small or shrunken seeds due to the necessity of using all, or almost all, seeds from the characteristically poorly developed speltoid plants. Further, no evidence is presented that the seeds from  $\beta$  or  $\gamma$  speltoids were themselves of the  $\beta$  or  $\gamma$  speltoid constitution. In any case, in his argument from these data that differential elimination of speltoid zygotes borne on het speltoids helps to explain the  $\beta$  and  $\gamma$  ratios, Uchikawa fails to distinguish between a direct effect of genetic constitution on the viability of the zygote and the possibility that the constitution of the parental plant affects the viability of any, or all, seeds borne by it. It is unlikely that speltoid seeds borne by poorly developed speltoid plants are as viable as speltoid seeds produced by vigorous het speltoids, yet some of his calculations could be taken to indicate that they are more viable.

Lindhard (16) thought that the weights of seeds borne by het speltoids were correlated with their genetic constitution. His data are very striking but not consistent with any simple correlation or hypothesis. We have weighed and

sown separately the largest, smallest, and medium grains of two  $\beta$ , two  $\gamma$ , and six  $\alpha$ - $\gamma$  het speltoids. The test involved 468 seeds and no correlation was found between seed weight and segregation ratios.

Differential survival of the three classes of segregates in the field was postulated by Lindhard, Philiptschenko, and Uchikawa. Involved in their reasoning is the fact that  $\beta$  and  $\gamma$  (or C) speltoids are generally smaller and weaker plants than their normal and het speltoid sibs. But in all cultures recorded except Lindhard's, het speltoids are vigorous plants, taller than and having as many, or almost as many, culms as their normal sibs. Lindhard (16) used plant weights as an additional measure of vigor. In the first year of his experiments, 1918, he found the average weight of normals and het speltoids to be 63 and 36 gm. However, in later years, his results were very variable. For example, in one family comprising 33 normals and 247 het speltoids, grown in 1921, the respective weights per plant were 25 and 52 gm., while in one related family, also of Series  $\beta$ , they were 77 and 48. In a related  $\gamma$  family they weighed 60 and 46 gm. while in an  $\alpha$  strain the normals, het speltoids, and speltoids averaged 57, 84, and 7 gm. each. Such a disproportionately small size of the speltoids is atypical for Series  $\alpha$  and more like that for  $\beta$ , or  $\gamma$ . Lindhard remarked on the discontinuous variation in weights that occurs throughout his sublines of speltoids. On quite other bases it has been shown (12) that there is strong reason to suspect that some of Lindhard's "speltoids" were really hybrids with *T. spelta*, or its derivatives. That assumption might well explain these weight variations, as well as many other puzzling features by which Lindhard's results differ from those of other workers. Some varieties of *T. spelta* have more slender straw and weigh less than most varieties of *T. vulgare* and we know from speltoid data that there are size and vigor genes in the C chromosome of the latter. Crossing over between C and its homoeologue from *T. spelta* would suffice to explain the more random association of weight with other characteristics in later generations.

The heights and numbers of culms have been recorded for practically all plants in our cultures and many have been weighed. In none of our strains of any of the three Series are het speltoids shorter or of smaller average weight than their normal sibs when comparisons are made within one season. In Series  $\beta$  and  $\gamma$ , speltoid plants are characteristically smaller and weaker than their normal and het speltoid sibs. It would not therefore be surprising if under adverse growing conditions they should be differentially eliminated. This might apply especially to fall-sown strains such as Lindhard's and some of Åkerman's, and Nilsson-Ehle's. Following late sowing and a very severe winter Åkerman (1) found 12.8% survival from normals and only 3.5% from their  $\gamma$  speltoid sibs. Following two mild winters, the survival rate was 55 to 57% from sowings of normals and speltoids alike. It is usually higher for spring sowings. Though Uchikawa (36) postulates differential elimination of speltoids to explain  $\beta$  and  $\gamma$  ratios, his Tables 11 and 12 show

almost perfect survival from crosses between het speltoids and speltoids which give a high proportion of speltoid progeny. He does not give survival rates for the progeny of het speltoids.

Our progenies of  $\beta$  het speltoids are heterogeneous, by statistical test, for survival to maturity as well as for emergence. This heterogeneity is not, however, reflected in heterogeneity of segregation ratios within or between seasons or strains.

The factors which cause the fluctuations in emergence and survival cannot be shown to act differentially on the three classes of segregates in our cultures. The tests do not rule out the possibility that there has been a slight and fairly constant greater elimination of speltoid segregates, but, as will be shown, this cannot be an important factor in determining segregation ratios or the differences between the three Series, since it is overshadowed by the factors of gamete formation and functioning. In the absence of proof of differential zygotic elimination, the ratios determined at plant maturity under any ordinary conditions of growth give the best available estimate of the frequencies with which the three classes of zygotes are formed by het speltoids.

#### *E. Spore and Gamete Formation*

##### *(a) Series $\alpha$ and $\beta$*

In Series  $\alpha$ , it may be assumed that mutant and normal gametes are formed in equal numbers. In Series  $\beta$ , an excess of mutant gametes, which function on the female side, must be postulated. An estimate of the proportions of 20- and 21-chromosome pollen formed by  $\beta$  het speltoids is readily obtained from counts of micronuclei in the pollen tetrads. During the first meiotic anaphase, the component chromatids of the univalent C chromosome characteristically separate and pass towards opposite poles. They may reach the poles in time to be included in the dyad nuclei, or either one, or both, may be left out on the spindle. During the second meiotic division, these "daughter univalents" do not ordinarily divide again. If they have been included in the dyad nuclei, they may go to either pole of the second division spindle. If they have been left out in the dyad cytoplasm, they can, apparently, be picked up during the second division. In either case, they may either be left out in the cytoplasm, or included in the tetrad nuclei at the end of the second meiotic division. If left out, they apparently take no further part in gamete formation, but form micronuclei. The presence of two, one, or no micronuclei in a pollen tetrad therefore indicates that it will form four, three, or two gametes, respectively, with only 20 chromosomes each. Deviations from this expectation will, of course, occur if chromosomes other than the univalent C fail to pair and segregate normally. We have found this to occur in varying frequencies up to as high as 6% of the pollen mother cells. The calculated proportion of 20-chromosome, speltoid-producing gametes will therefore be an overestimate.

From one het speltoid of strain  $\beta 1$ , two of  $\beta 4$ , and one of  $\beta 5$ , samples of 150 pollen tetrads each were examined and 96 tetrads from a  $\beta$  het speltoid that



arose from a subcompactoid. The proportions of 20- : 21- chromosome male gametes they would be expected to produce were 84 : 516, 99 : 501, 65 : 535, 82 : 518, and 30 : 354, i.e., 14.0, 16.5, 10.8, 13.7, and 7.9%, respectively of the pollen would be normal. Uchikawa (36) from counts of 604 tetrads calculated that 18.9% of the pollen is normal in his  $\beta$  het speltoids. He implies that variability was encountered, see below, but gives no data from which its extent can be determined.

In view of the low frequency with which 20-chromosome pollen usually functions when in competition with an abundant supply of normal pollen (see next section), the determination of the frequency with which it is formed is of less direct significance than it is of indirect value in giving some indication of the relative proportions in which mutant and normal ovules are formed. As shown above, both types of ovules must be functional since there are no more sterile florets on het speltoid than on normal spikes. It is, unfortunately, too laborious an undertaking, especially in view of the probable variability, to determine directly on a statistically significant scale the proportions of mutant and normal ovules. Watkins (38, 39) found that in pentaploid hybrids there was no evidence of any appreciable difference in the frequency with which univalents are lost during androsporogenesis and gynosporeogenesis. He pointed out that while chromosomes left out of the dyad nuclei and picked up by the transverse second division spindle of androsporogenesis have a chance of getting into a functional pollen grain, the same does not hold during ovule formation. In gynosporeogenesis the two spindles have the same orientation and chromosomes left out of the dyad nuclei are therefore not likely to be carried into the innermost, potentially functional, gynospore. There may therefore for this reason be a somewhat greater proportion of 20-chromosome ovules formed than of pollen grains, but as Watkins pointed out for his hybrids that had seven univalents, the difference is hardly likely to be significant in affecting progeny ratios that may be influenced by so many other factors. This should be even truer for  $\beta$  het speltoids that have only one univalent, and since we do not know what other factors may influence its inclusion or loss, we can only proceed on the assumption that it is similar in gynosporeogenesis to that determined from pollen tetrad counts and see how closely this assumption fits the genetic data.

Quite unexplained is Uchikawa's report that a 41-chromosome speltoid of the constitution  $40 + C^{\alpha}$  gave 42-, 41-, and 40-chromosome speltoids in the ratio 1 : 1 : 0.1. Unless natural crossing or some other chromosome aberration is involved, this ratio would indicate that the mutated univalent,  $C^{\alpha}$ , is included or left out of the gynospore nuclei with equal frequency, whereas a normal C chromosome when univalent in a 41-chromosome het speltoid gets left out 3 to 5 times as often as it gets included.

#### (b) Series $\gamma$

There are more indeterminables in  $\gamma$  than in  $\beta$  het speltoids. When the normal C chromosome and the C lacking a segment, "Cd" or "Cts", are

associated at metaphase they presumably segregate normally giving equal numbers of normal and mutant spores. When unassociated at first meiotic metaphase they both behave like the univalent C of  $\beta$  het speltoids and from none to all four of the "daughter univalents" may therefore get into, or be left out of, any of the tetrad nuclei. When there is a micronucleus in each quadrant of the tetrad, there are obviously (apart from the possibility of chromosomes other than C being left out) four spores with 20 chromosomes each. If there are three, then three spores have 20 and one has 21. When there are only two micronuclei, their relative position has to be considered. If they lie in diagonally opposite quadrants, there are two spores with 20 and two with 21. If they are in adjacent quadrants, two "daughter univalents" may have been included, one in each, or both in one of the other quadrants. Observations were, when possible, made in the brief stage after formation of the nuclear membranes in the tetrad and before the dyad arrangement is obscured by the development of the secondary, transverse walls. If observations are made after the tetrad walls are fully formed, the assumption may be made, for purposes of calculation, that micronuclei in adjacent quadrants had previously been in the same or different dyads with equal frequency. When there are no micronuclei, all quadrants may have 21 chromosomes, as expected if C and Cd or Cts have been paired, or, if they were unpaired, some gametes may have 22 and others only 20 chromosomes.

In het speltoids of strain  $\gamma$  2 the C and Cd chromosomes were unpaired in 13.6% of 154 first metaphase pollen mother cells. In 372 tetrads only 9.4% had micronuclei. Assuming these to be representative samples and all micronuclei to be constituted of C or Cd chromosomes, 4.2% of the normal tetrads resulted from pollen mother cells with C and Cd unpaired and their nuclei with 20, 21, and 22 chromosomes comprised 1, 2, and 1% respectively of the total sample of spores. From this, strain  $\gamma$  2 het speltoids are calculated to produce 20-, 21-, and 22-chromosome pollen in the proportion of 6.1 : 92.4 : 1.5. Details of the method of calculation were given by Smith (33, 34). It may be assumed that the 92.4% of 21-chromosome pollen comprises equal numbers with 20 + C and 20 + Cd, and similarly the 1.5% comprises 21 + C and 21 + Cd. The foregoing all assumes, of course, that C and Cd univalents have similar chances of inclusion.

With so many possible variables, the only important conclusion to be drawn on Series  $\gamma$  is that 20-chromosome pollen is formed sufficiently often to require consideration in the interpretation of the ratios. Used as an indication of the frequency with which 20-chromosome ovules are formed, it gives an estimate of the frequency with which  $\beta$  het speltoids may be expected to arise from  $\gamma$  het speltoids and this can be compared with the observed frequency in various strains.

#### *F. Certation*

Certation, the measure of competition between diverse pollen grains in effecting fertilization, is evidently of great significance in determining the

$\beta$  segregation ratios. Crosses of normals or speltoids with het speltoids provide a measure of the frequency with which the mutant and normal pollen grains function, but not, of course, directly a measure of their relative innate capacity to function, since we have first to take into account the frequency of formation and secondly to recognize that the total amount of pollen available, as well as many other environmental factors, may influence the effective competition.

(a) Series  $\alpha$  and  $\gamma$

No definite estimate can be made of the degree of certation in Series  $\alpha$  for the reason that  $\alpha$  grades into  $\gamma$ . By definition, "good"  $\alpha$  strains give ratios approaching 1 : 2 : 1 though almost invariably with some shortage in the numbers of het speltoids and especially of speltoids. Certation can therefore be occurring to only a limited extent in them. The theoretical limit of certation would be reached in  $\gamma$  het speltoids that give 1 : 1 : 0 ratios. Some strains closely approach this limit.

Nilsson-Ehle (24) showed that in a  $\gamma$  strain which gave 135 normals, 138 het speltoids, and no speltoids on selfing, the cross of het speltoid by normal gave nine normals and seven het speltoids, while the reciprocal cross gave 29 normals only. When in later generations a very weak speltoid segregate was obtained, Nilsson-Ehle (23) related the degree of weakness of the homozygous mutants to the degree of incapacity of the mutant pollen to effect fertilization. In a strain intermediate in our classification between  $\alpha$  and  $\gamma$ , he obtained five normals and five het speltoids from the cross het speltoid by normal and 10 normals to one het speltoid from the reciprocal cross. Åkerman (1) obtained 126 normals and 22 het speltoids from crossing normals with het speltoids. Sib het speltoids on selfing gave 45 normals, 53 het speltoids, and three speltoids. He therefore classified this as Series A, while we call it  $\gamma$ . In any case, it is of the chromosome type that *forms* mutant and normal gametes in approximately equal proportions. The ratio obtained from the cross therefore indicates that normal pollen in this case had almost a 6 : 1 advantage over mutant pollen. Crosses of normals by our  $\gamma$ 4 het speltoids gave 72 normals and three het speltoids (Table XII). In this case, normal pollen had a 24 : 1 advantage. Uchikawa's (36) crosses of normals by het speltoids derived from our  $\gamma$ 4 strain gave 76 normals and eight het speltoids, while his crosses of speltoids by their het speltoids gave 52 het speltoids and six speltoids. Together, these crosses show the normal pollen having a 9 : 1 advantage.

(b) Series  $\beta$

Crosses of speltoids by  $\beta$  het speltoids were first made by Nilsson-Ehle (24) who obtained nine het speltoids and no speltoids, from which he concluded that the normal pollen of  $\beta$  het speltoids has a very great competitive advantage and that this is very important in determining  $\beta$  ratios. This was confirmed by Lindhard (16) who from crosses of normals by  $\beta$  het speltoids got 103 normal and no het speltoid progeny. From the same cross in strain  $\beta$ 4, we got 91 normals and no het speltoids.

TABLE XII

## RECIPROCAL CROSSES

Female parent	Male parent	Seeds sown	Seeds germ'd	Percentage progeny			Total matured	Percentage gross survival
				N	Het	Sp		
$\beta$ 4 het	N	108	87	25.9	74.1	0.0	85	78.7
$\beta$ 4 het	Selfed	275	230	21.2	78.8	0.0	231*	83.6
$\beta$ 4 N	$\beta$ het	100	91	100.0	0.0	0.0	90	90.0
$\gamma$ 1 het	N	21	20	50.0	50.0	0.0	20	95.2
$\gamma$ 1 het	Selfed	192	167	42.2	52.6	5.2	154	80.2
$\gamma$ 4 het	N	75	51	55.3	44.7	0.0	47	62.7
$\gamma$ 4 het	Selfed	252	207	47.3	52.2	0.5	205	81.3
$\gamma$ 4 N	$\gamma$ het	86	75	96.0	4.0	0.0	75	87.2

\* Including one dwarf, unclassifiable plant.

All the above plants were grown in 1929.

Uchikawa (36) on the other hand, found a much smaller degree of certation. From crosses of normals by  $\beta$  het speltoids derived from our  $\beta$ 4 strain, he got 92 normals and 14 het speltoids, which indicates that mutant pollen effected 13.2% of the fertilizations. From crosses of  $\alpha$ ,  $\beta$ , and  $\gamma$  speltoids by  $\beta$  het speltoids he got 38 het speltoids and seven speltoids, indicating 15.6% fertilization by mutant pollen. These are higher rates of functioning than in his own self-fertilized  $\beta$  het speltoids, as judged from their proportion of speltoid segregates. Uchikawa believes that in selfed  $\beta$  het speltoid the mutant pollen actually functions as well as in his crosses, but that about 94% of the resultant  $\beta$  speltoid progeny are eliminated. We shall consider two other possibilities:

(1) That Uchikawa used small amounts of pollen in his crosses, relative to that available in selfings, and thus reduced competition for the mutant gametes.

(2) That a change has occurred in his strain analogous to though not as extreme in its genetic effect as that which has a number of times produced "modified  $\beta$ " from het fatuoids (27, 31, see also 5). Uchikawa recorded that in 18,357 p.m.c. of  $\beta$  het speltoids there were  $20_{II} + 1_I$  and in only 122 were there any other abnormalities. In modified  $\beta$  het fatuoids there are  $20_{II} + 1_I$  as in  $\beta$  het fatuoids or  $\beta$  het speltoids and we have so far been able to find no definite cytological difference. But whereas in  $\beta$  het fatuoids the mutant, 20-chromosome, pollen is very largely nonfunctional, it is highly functional in modified  $\beta$  het fatuoids.

Uchikawa's  $\beta$  speltoid segregates also differ from those of most other workers in being partially fertile instead of almost completely sterile. The "nulli IX", 40-chromosome  $\beta$  speltoids that Sears (32) has obtained in his current analysis are as sterile as ours. He reports (personal communication) "I have grown several dozen nulli-IX plants over a period of years, and to my knowledge not a seed has set on any one of them from self-pollination, in spite of reasonably

good female fertility. As a matter of fact, indehiscence of anthers is the rule, although I cannot say definitely that an occasional anther does not dehisce."

All of the vigorous, more or less fertile, speltoids arising sporadically from Series  $\beta$  which we have examined have chromosomes other than C in excess numbers. Sears (32) has shown that normal pollen has no competitive advantage over pollen lacking chromosome II when it has chromosome XX in excess. This compensation phenomenon applies in varying extent to other chromosomes, from which it follows, as Sears points out, that there will be a selection in favor of compensating combinations. Since Uchikawa found only 40 chromosomes in an extraordinary number of p.m.c. it cannot be whole chromosome compensations that are involved in his  $\beta$  speltoids, but it could be a translocated segment.

### G. *Correlation of Genetic with Cytological Data*

Attempts to compare observed genetic ratios with theoretical expectations based on chromosome loss in pollen tetrads have been made by Smith (34) and by Uchikawa (36). Further analyses have now been made of Series  $\beta$  and of  $\alpha$  and  $\gamma$  jointly.

#### (a) *Series $\beta$*

Smith's original estimate (34) of the frequency with which 20-chromosome pollen functions was based on the frequency of speltoids found in the progeny of selfed het speltoids. It was concluded, from the fact that the expectation based on this and the tetrad counts was very close to the ratio observed, that it is unnecessary to assume zygotic elimination of speltoids. The speltoids, however, comprise only about 1% of the progeny, which makes it unwise to base expectation on them. Further, statistical tests have since shown that the tetrad counts are heterogeneous.

Uchikawa's (36) argument, in brief, is: Pollen tetrad counts show that 21- and 20-chromosome pollen grains are produced in the ratio of 1 : 4. Crosses of het speltoids by normals show that the ovule proportion is the same. The cross of normal by  $\beta$  het speltoid gave 15 normals and three het speltoids; thus 21- and 20-chromosome pollen is functioning in the ratio 1 : 0.2. Hence, on selfing  $\beta$  het speltoids a ratio of 1 N : 4.2 Het : 0.8 Sp is expected. The observed ratio was 1 : 4.5 : 0.05 and the discrepancy is accounted for by the assumption that 15/16 of the speltoid offspring (12.5% of the total zygotes) are eliminated before reaching maturity. His general conclusion is that the ratios are due: (a) to a 4 : 1 ratio of 20- and 21-chromosome gametes, (b) to certation favoring normal gametes in the proportion of 20 : 1, and (c) zygotic elimination of speltoids. The fact (a) that the ratios vary considerably and (b) that according to his own statement "frequency of chromosome elimination is almost necessarily affected by various environmental conditions" does not appear to have been considered sufficiently in reaching these conclusions.

Variation in the proportion of normal and mutant ovules is apparently the only factor that could appreciably affect the ratio of normals to het speltoids. For instance, if one accepts Uchikawa's figure of 1 : 4 for ovules, but changes the assumed ratio of functioning normal and mutant pollen from 1 : 0.2 to 1 : 0.02, the expected zygotic ratio would be changed only from 1 : 4.2 : 0.8 to 1 : 4.02 : 0.08. If instead of 1 : 0.2 functioning one assumes 1 : 0.0125, the zygotic ratio expected would be 1 : 4.0125 : 0.05. In other words, if the ovule formation ratio remains the same, a change in the assumed frequency of functioning of mutant pollen from 1/5 to 1/80 of that of normal pollen would change the expected ratio of normals: het speltoids only from 1 : 4.2 to 1 : 4.0125. This latter change is insignificant when compared to the variations in N : H ratios that have to be accounted for. On the other hand, the change in the assumed frequency of functioning mutant pollen would reduce the expectancy for the speltoid class by 15/16.

The assumption of great differential elimination of speltoid segregates, which is an integral part of Uchikawa's conclusions, seems unnecessary on the basis of his own, Sears', or our data. Sears (32) found 1.5% speltoids in a population of 134 plants from 135 seeds. Certainly zygotic elimination after the stage of seed formation was not a significant factor in this case. On the other hand, variations in certation especially when chromosome deficiencies or aberrations are involved, are well known to be affected by differences in the amount of pollen applied and by the nature of the deficiency or other aberration in the chromosome primarily concerned. We conclude therefore that the frequency of speltoids obtained from  $\beta$  het speltoids depends: (a) largely on the degree of certation between 20- and 21-chromosome pollen, (b) to a much lesser degree on zygotic elimination, and (c) very little on the proportion of 20- : 21-chromosome pollen formed, since there is always a great excess of the former.

The primary characteristic of  $\beta$  ratios, the great excess of het speltoids (1 : 3, or usually more, instead of the 1 : 2 on Mendelian expectation) depends jointly on certation and the fact that female gametes with 20 chromosomes are formed three or more times as often as normal ones with 21 chromosomes, and are functional. Sears found the female transmission rate of nullisomics to be about 75% regardless of the chromosome concerned. In  $\beta$  het speltoids it is commonly somewhat higher than this. The fluctuations in the proportion of normals and het speltoids derived from self-fertilized het speltoids are predominantly due to the frequency with which the univalent C chromosome is left out of the gynospore during meiosis.

As for the use of pollen tetrad data in calculating the probable proportion of 20- and 21-chromosome gynospores, it has to be said that although the ratios are thereby reasonably well fitted there are some ratios that indicate a higher proportion of normal ovules than are estimated from any of our, or Uchikawa's, tetrad counts. This may mean that the C univalent is actually lost less often in gynosporogenesis. The discrepancy could, however, be

explained either as resulting from the variability of the tetrad counts, or to their giving an overestimate of C-chromosome loss because other chromosomes may have formed an appreciable proportion of the micronuclei counted.

(b) *Series  $\alpha$  and  $\gamma$*

From crosses of Series A (our  $\gamma$ ) het speltoids by normals, Åkerman (1) obtained 25 normals and 25 het speltoids. From similar crosses in strains  $\gamma 1$  and  $\gamma 4$  we got 26 and 21. Uchikawa (36) got 9 : 7, 17 : 15, and 16 : 14 in three crosses. From three crosses of  $\gamma$  het speltoids by speltoids he got seven het speltoids : six speltoids, 11 : 9, and 9 : 7. Together, these consistent results show that 120 normal and 104 mutant ovules produced mature plants. We may conclude that this represents approximately the proportion in which normal and mutant ovules are formed. If univalent loss is similar in ovules and pollen, our tetrad studies would give an expectation of 6.1 ovules with 20 chromosomes: 46.2 with 20 + Cd or Cts: 46.2 with 20 + C: 1.5 with 20 + C and an additional Cd or Cts chromosome that would not affect the phenotype in the presence of two C chromosomes. The cytologically "expected" proportion of "normal" and "mutant" ovules is therefore 47.7 : 52.3. In view of the variability in tetrad counts, the unknowns in the interpretation of micronuclei as indicators of gametic types formed in Series  $\gamma$  and the varying certation ratios shown in crosses with  $\gamma$  het speltoids as the pollen parent, no closer numerical agreement could be expected.

## V. The Nature of the Speltoid Mutations

Series  $\beta$  het speltoids arise through loss of a C chromosome (in Sears' terminology they are "monosomic IX"). The missing chromosome carries factors essential to the expression of the "beardless normal" type of *T. vulgare*. As Watkins and Ellerton (41) have emphasized, the term "beardless" is a misnomer, but it is in general use. True beardless wheats occur but are not widely cultivated. There are two genetically different tip-awned types. "Tipped I" is the "beardless" type commonly grown in Europe and America and considered herein. The gene determining its type of tip-awn is on the C chromosome and is denoted  $B_1$ . Tipped 2, found chiefly in Asiatic wheats, is determined by a gene designated  $B_2$ . This symbolism is used to imply that the B genes are phylogenetically alleles, but now on different, probably homoeologous, chromosomes in these polyploids. The awn formula of European "beardless" wheats is  $B_1B_1;b_2b_2$ ; that of the Asiatic is  $b_1b_1;B_2B_2$ . True beardless wheats are  $B_1B_1;B_2B_2$ , and  $b_1b_1;b_2b_2$  is fully bearded. The gene or gene complex k (Watkins (40)) which determines "normal" *T. vulgare* glume shape and thickness is also on the C chromosome 27 or more units from the B locus. The C chromosome may therefore be symbolized as ( $B_1-27+—k$ ) in ordinary "beardless" wheats and as ( $b_1-27+—k$ ) in bearded varieties. Since Series  $\gamma$  het speltoids arise through loss of all or part of the long arm of the C chromosome, genes  $B_1$  and k must be located in it. This long arm is marked by a secondary constriction about three-eighths

along its length. Chromosome configurations in het speltoids lacking part of the long arm, Fig. 12*a, b*, demonstrate that the deficiency, and therefore the segment  $B_1-k$ , is interstitial in the distal segment.

Bearded is definitely recessive, though to varying degrees in different crosses. The glume characters are intermediate in the heterozygote and opinions have differed whether speltoid ("K" according to Watkins) should be considered recessive or dominant. Nilsson-Leissner (26) pointed out that while the speltoid mutation as a whole may be considered recessive, in detail only beards are fully recessive. The head-type is nearer dominant and the glumes are intermediate. The "complex" mutations, he said, revealed the inadequacy of the dominance concepts then current. Lindhard (16) pointed out that those concepts derived from a too literal acceptance of the presence and absence hypothesis.

The genetic evidence makes it highly probable that a deficiency is involved in Series  $\alpha$  as in  $\gamma$ —*contra* Uchikawa (36) and others who class  $\alpha$  speltoids as gene mutations. Selfed het speltoids that have arisen at one step from the normal, i.e. by "total mutation", always give ratios that deviate in some degree from 1 : 2 : 1 towards 1 : 1 : 0, thus indicating reduced functioning of the mutant pollen. Nilsson-Ehle (25) showed further that in the "total-mutations" of any Series the interstitial region between the beard and glume factors must in some way be affected for no crossing over takes place between them, though it does in bearded speltoids that have originated by segregation following the crossing of part-mutants, i.e., beardless speltoids and bearded normals. He found 27 to 36% crossing over in studies of part-mutants, which is similar to the 28 to 39% found by Watkins in intervarietal and interspecific crosses which involved the  $B_1$  and  $k$  loci. The ratios from selfed part-mutants do not usually deviate appreciably from 1 : 2 : 1. These data are all explicable on the assumption, adumbrated initially in 1921 by Nilsson-Ehle, that the total-mutation, whatever its Series, involves loss of not less than the  $B_1-27+—k$  region of the C chromosomes. The extent of the loss—a microscopically indeterminable or doubtfully determinable segment in  $\alpha$ , a readily determinable segment which may be as large as the entire longer arm in  $\gamma$  and loss of the entire chromosome in  $\beta$ —evidently determines in the strains here considered the degree to which chromosome behavior is affected in meiosis and also the extent to which mutant pollen is handicapped in competition with the normal in het speltoids.

Uchikawa's data on the segregation of  $F_1$  hybrids between  $\alpha$  and  $\gamma$  speltoids indicate Cts pollen is very little handicapped when in competition with  $C^\alpha$ . Such  $F_1$  speltoids having one  $\alpha$  and one  $\gamma$  C chromosome ( $C^\alpha$  Cts in this case) give only speltoid progeny but they are of three chromosome types,  $C^\alpha C^\alpha$ ,  $C^\alpha$  Cts, and CtsCts, in the ratio 1 : 2 : 0.6. In contrast, Cts pollen is severely handicapped when in competition with normal (C) pollen, for selfing CCts het speltoids gives a 1 : 1 : 0.05 ratio of CC, CCts, and CtsCts progeny.

In 1914, before it was known that *T. vulgare* is a polyploid species, Nilsson-Ehle conceived the normal phenotype as resulting from a balance of factors



tending to cause development on the one hand towards *T. spelta* in glume and spike characteristics and on the other towards *T. compactum*. Winge in 1924 fitted this concept to the polyploid status of these three species—see Huskins (11). The concept of Philiptschenko (29, 30) was superficially similar. He considered that a gene C thickens and shortens the ear while S produces lengthening: *T. compactum* is CCss, *T. spelta* ccSS, and *T. vulgare* ccss. The genes on his concept work as inhibiting factors affecting various parts of the developmental process.

The present cytogenetic data from speltoids and compactoids can most simply be fitted by the concept that factors  $B_1$  and  $k$  are inhibitors or suppressors which in normal wheat counteract the effect of awn-producing and glume-thickening genes located on chromosomes other than C.\*

If a bearded spelt-like form is considered the original wild type from which *T. vulgare* has been derived by mutation, then Watkins' factors  $k$  and  $B_1$  are mutations of  $K$  and  $b_1$  which produce the cultivated glume and tip-awn type. To use this formal genetic concept and yet recognize that deficiency of  $k$ , as in mutant speltoids, gives a phenotype similar to that produced by  $K$  and that the absence of  $B_1$  gives approximately the same effect as the presence of  $b_1$  it is only necessary to call  $K$  and  $b_1$  "amorphs" (Muller (22)). Sears (32) has shown that  $b$  is almost though not quite an amorph since the nullisomic beardless speltoid, -- ;  $B_2B_2$ , has slightly longer awns than the normal, which is  $b_1b_1; B_2B_2$ , in his variety Chinese Spring. The polyploid nature and probable hybrid ancestry of *T. vulgare* help to make the inhibitor concept more acceptable than it might be in a diploid, but it must of course be recognized for the present as a formal genetic, not a physiological, interpretation.

In the present analyses of speltoids there is no way of determining where the "positive" speltoid glume and beard factors are located or whether they are single factors that in the ancestral diploid form, or forms, were true alleles of the present day inhibitors. Alternatively the beard and speltoid glume characters could be the expression of many genes, that is, of the "genetic background" of *T. vulgare*. Watkins' evidence indicates that different alleles distinguish various species and his symbolism implies that the  $K$  alleles are, through polyploidy, on homoeologous chromosomes. His formulae are:

<i>T. vulgare</i>	$kk; KK; K^dK^d$
<i>T. spelta</i>	$K^*K^*; KK; K^dK^d$
Speltoid	$KK; KK; K^dK^d$

This speltoid is not a mutant but the type that segregates from crosses of *T. vulgare* with *T. turgidum*. The three pairs of group symbols represent, of course, parts of three homoeologous pairs of chromosomes, present on account of the hexaploidy.  $K^*$  is the spelta gene,  $K$  the speltoid, and  $K^d$ , which may

\* McFadden and Sears (*J. Heredity*, 37 : 81) believe that the *T. vulgare* inhibitors of spelta characteristics may have been acquired by transfer of a block of genes from a Lake Dweller wheat into *T. spelta*.

be identical with  $K^s$ , the gene producing the somewhat speltoid-like glumes of *T. dicoccum*. Speltoids involving deficiency would on this symbolism be -- ; KK;  $K^dK^d$ . Those compactoids which involve the presence of an extra pair of C chromosomes would be kk; kk; KK;  $K^dK^d$ , while those that have a pair of isomorphic chromosomes, each constituted of two major arms of C, are kk kk; KK;  $K^dK^d$ .

Winge (42) assumed that a specific chromosome, B, carries the beard and speltoid glume factors. We can find no evidence for this nor can Sears (personal communication) though he now has trisomes for most of the 21 chromosomes. Many chromosomes seem to have an effect in producing or lengthening beards and in hardening the glumes. Polysomics II and XX have fairly stiff glumes, I and XVII have glumes less tough than normal, XV has shorter glumes (as has the speltoid) VI, X and XIX have broader, and probably tougher glumes, while in V and XVIII they are larger. Sears' evidence, then, favors the assumption that the speltoid phenotype is due to many genes—perhaps it is nearer the original wild type—and that the beardless cultivated type is determined predominantly by the two genes  $B_1$  and k in European or  $B_2$  and k in Asiatic wheats. Once such inhibitors arose they would naturally be selected by man because they produce a phenotype highly desirable agriculturally and would soon come to be part of the normal genotype of cultivated wheats.

It is an open question whether the speltoid mutation occurs oftener than other mutations in wheat, or is merely picked out more often on account of its striking characteristics. If the latter, it would be logical to assume that chance irregularities of mitosis and meiosis, affecting at random any chromosome, are responsible for the occurrence of  $\beta$  het speltoids. Those  $\gamma$  het speltoids which have lost the entire long arm of C could likewise be due to chance irregularities. Telokinetic chromosomes arise through misdivision of the kinomere in unpaired chromosomes, as do the isochromosomes that produce Type I subcompactoids. Unpaired chromosomes occur frequently in some varieties of wheat newly developed by hybridization (Hollingshead (7), Love (20, 21) ), as do speltoids. It seems necessary, however, to assume some more specific and recurring abnormality as responsible for the interstitial deficiencies of  $\alpha$  and most  $\gamma$  het speltoids. One obvious possibility is homoeologous pairing of the type BC postulated by Winge (42), but followed by double crossing over and hence substitution for the sector including  $B_1$ —k of one lacking it, instead of by his whole chromosome substitution. It is not necessary to assume that B carries speltoid factors, only that it lacks genes  $B_1$  and k. The cytological evidence for homoeologous pairing of C after it has mutated (Winge and Huskins) is now known to be in large part faulty and at best slight. It is not, however, negligible and the evidence which Uchikawa (36) presents as disproving it actually favors the interpretation that when a normal C chromosome lacks a homologue it is likely to pair with some other chromosome, presumably a homoeologue. In  $\beta$  het speltoids he finds 0.31% trivalent formation, whereas in all other segregates of all Series,

the range is 0.08 to 0.2%. Multiple chromosome complexes are fairly common in "haploids" of the hexaploid wheats, but they occur also in true haploids of the diploid *T. monococcum* (Kihara and Katayama (15)) and in any case cannot be considered unequivocal evidence of homoeologous pairing, as interchange of segments also can produce them. Ellerton (4) observed chromosome catenation in the  $F_1$  of *T. sphaerococcum* Perc.  $\times$  *T. vulgare* and explained the high proportion of het speltoids found in  $F_2$  as due to factors  $K_2$  and  $k_1$  being on the chromosomes involved in the reciprocal translocations responsible for the catenation. This is probably the simplest explanation for the high frequency with which het speltoids arise in many intervarietal hybrids of *T. vulgare*. His assumption that reciprocal translocations may account for the differences in frequency with which pure lines of *T. vulgare* produce het speltoid mutants would appear, however, to be valid only if he implies also duplication as a result of segregation after interchange, in addition to that present initially through polyploidy. The occurrence of true breeding hybrids between polyploids and of "Shift" (3) gives genetic evidence of homoeologous pairing. Watkins (40) interprets the lack of segregation for the  $K$  and  $K^d$  factors in the cross *T. turgidum* and *T. dicoccum* in this way, but cytological confirmation is lacking.

A second possibility for which there is some cytological, and perhaps genetic, evidence is that the  $B_1$ - $k$  deficiency arises through crossing over in a duplicated region on C. There is evidence for duplication in the occurrence of occasional foldback pairing of C when it is univalent (Kerr, 13). Watkins (40) has evidence that the  $k$  gene is associated with something that affects pollen functioning. He suggests that this is a deficiency which is compensated for by a duplication on a member of a different genome. It seems that duplication on C and deficiency elsewhere would give the same result. If there is a duplication on C it is possible, though not essential to this interpretation of the origin of the  $B_1$ - $k$  deficiency, that it is of the  $B_1$  and  $k$  loci themselves, or of one of them. That region of C would then be similar to, but of greater extent than, the Bar locus in *Drosophila*, and deficiency would result from "unequal crossing over". Duplications of  $B_1$ , or  $k$  would, like the original mutation, be favored by human selection if they enhanced the inhibitory effect on awns and spelta-type glumes. Any possibly deleterious effect of duplication on pollen functioning would be less in a polyploid than a diploid and after a single generation, wheat being self-fertilized, there would be some plants homozygous for the duplication and in them there would be no competition with the original normal pollen.

The possibilities that may be envisaged to account for the  $B_1$ - $k$  deficiency are obviously too numerous for any one of them to be considered very seriously at the present stage. If they are borne in mind, evidence favoring or excluding them may be obtained. What is clear is that the speltoid mutation is not a gene mutation. Those speltoids that arise as segregates from crosses with *T. turgidum* may, of course, be differentiated by single loci rather than chromosome sectors, though Watkins (39) stresses that  $k$  and  $B_1$  are

probably gene complexes, not single genes, and Nilsson-Ehle concluded that even the part-mutations involve more than one gene. Watkins (40) attempted to clarify part of that issue by crossing his speltoids with *T. spelta*, but the  $F_2$  segregates were too difficult to classify. It is clear also that speltoids are not, characteristically at least, due to whole chromosome substitutions as originally thought by Winge and Huskins. Finally, it is clear that they are not simple (Mendelian) segregates from crosses between *T. vulgare* and *T. spelta*, though forms similar to mutant speltoids may arise in this way, and hybridization, even between varieties, of wheat does result in irregularities of meiosis, some of which may produce the speltoid mutation.

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## EXPLANATION OF PLATES

## PLATE I

- FIG. 23. Strain  $\beta$  1. Normal and het speltoid heads.
- FIG. 24. Strain  $\beta$  2. Normal, monosomic C (C-) het speltoid, and sterile nullisomic C (--) speltoid.
- FIG. 25. Strain  $\beta$  3. Normal-het speltoid chimera.
- FIG. 26. Strain  $\beta$  3. Normal, C- het speltoid, and anomalous fertile bearded -- speltoid which has an extra non-C chromosome and two fragments.

## PLATE II

- FIG. 27. Strain  $\beta$  4. Normal, C- het speltoid, and sterile dwarf -- speltoid.
- FIG. 28. Strain  $\gamma$  3. Normal and CC- deficient (CCd) het speltoid.
- FIG. 29. Strain  $\gamma$  1. Normal, CCd het speltoid, C- het speltoid, and CdCd speltoid.
- FIG. 30. Strain  $\gamma$  4. Normal, and CC-telokinetic short arm (CCts) het speltoid.

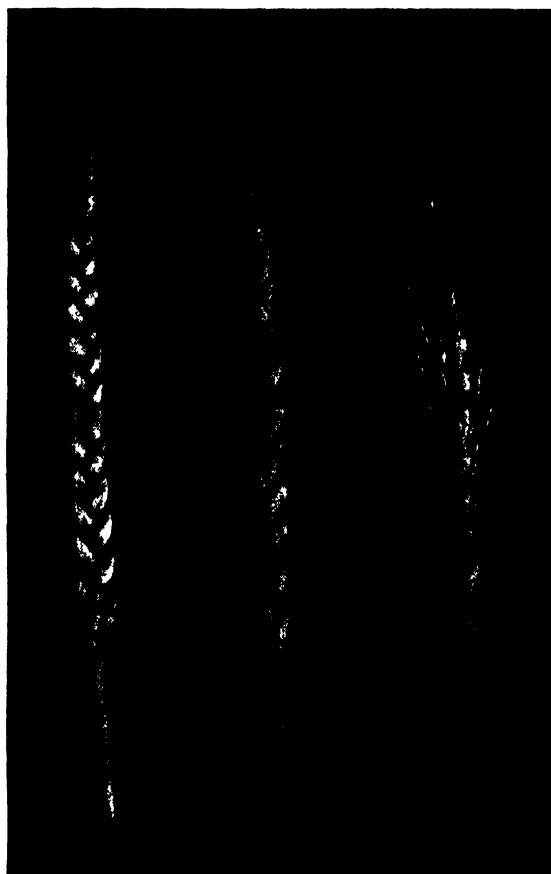
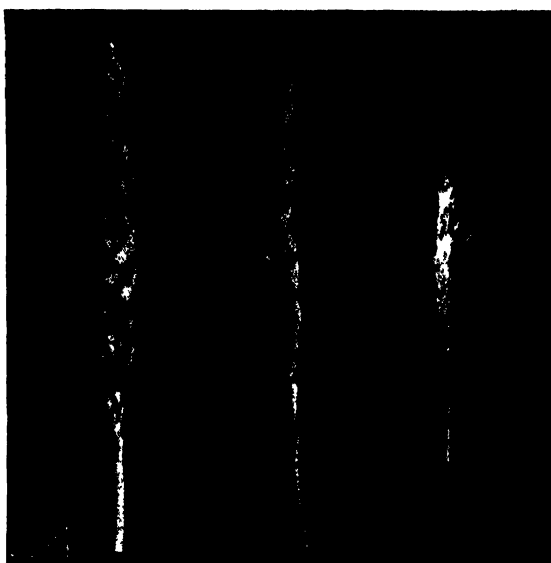
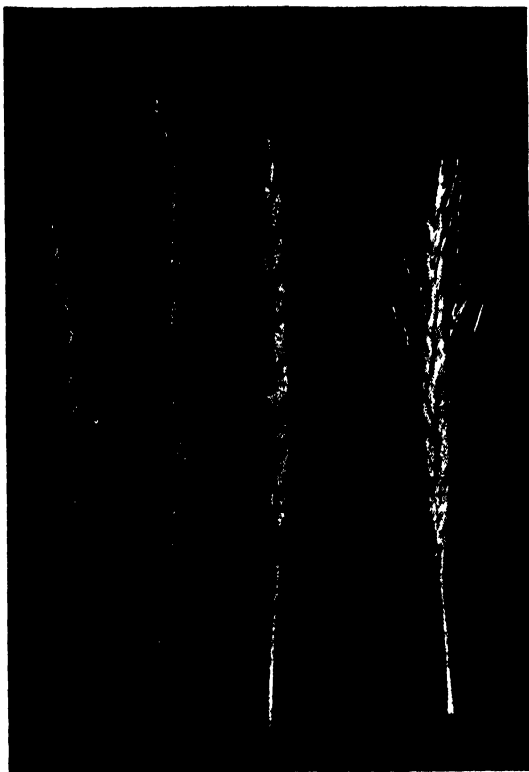
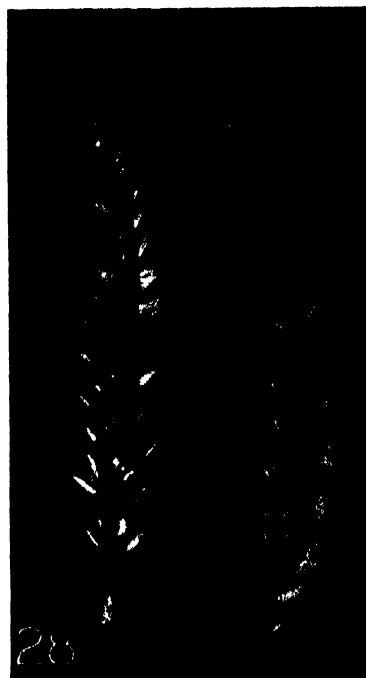
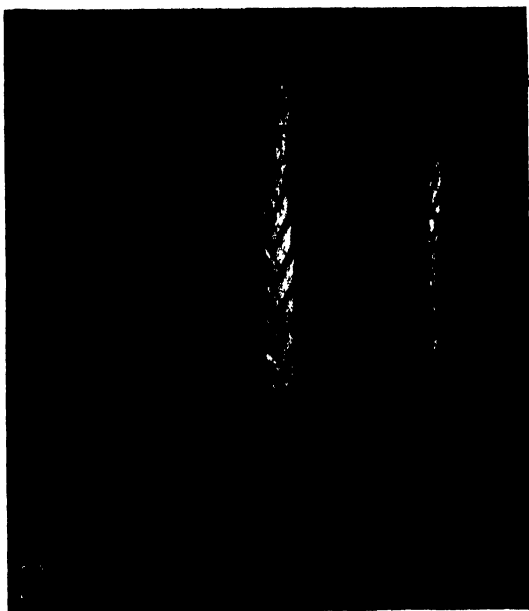
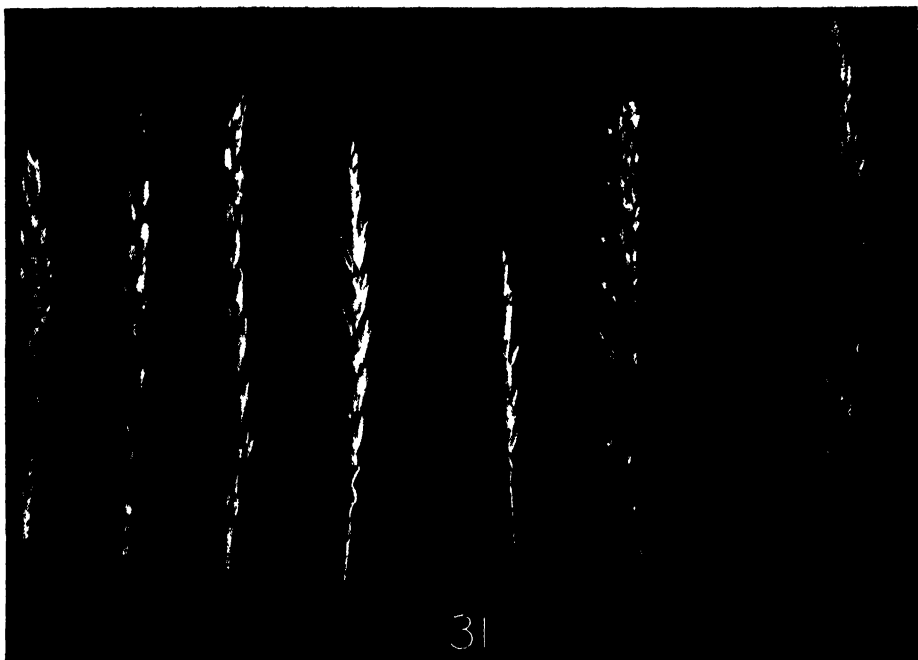
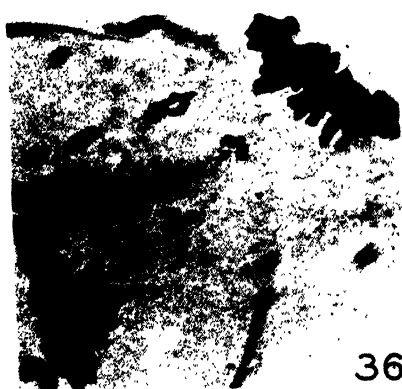


PLATE II

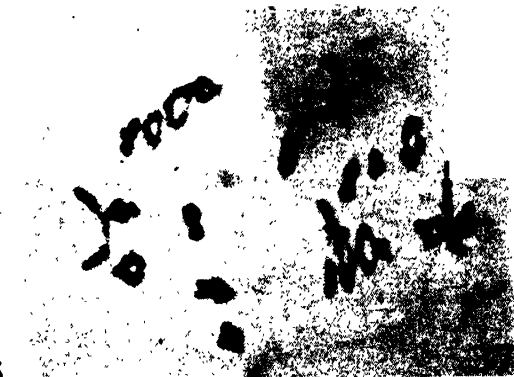




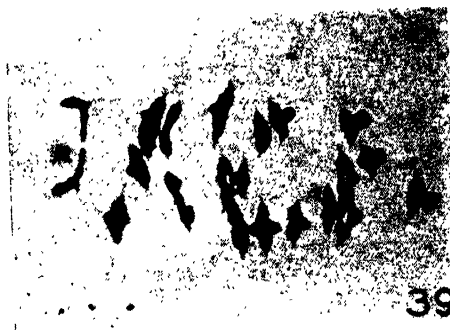




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## PLATE III

- FIG. 31. Strain  $\gamma$  2. Normal, CCd het speltoid, C- het speltoid, CdCd speltoid, Cd-speltoid, anomalous C- het speltoid with an extra non-C chromosome, anomalous--speltoid (lacking C but having an extra non-C chromosome).
- FIG. 32. Strain  $\alpha$  1. Normal, CC $\alpha$  het speltoid and C $\alpha$ C $\alpha$  speltoid.
- FIG. 33. Strain  $\alpha$  2. Normal, normal-het speltoid chimera, CC $\alpha$  het speltoid, and C $\alpha$ C $\alpha$  beardless speltoid.

## PLATE IV

*Photomicrographs of acetocarmine smear preparations of pollen mother cells.*

FIG. 34. Metaphase I, short-bearded het speltoid, strain  $\beta$ 2. Note univalent "C" chromosome ("C $_1$ ") off the plate.

FIG. 35. Metaphase I (chromosomes scattered by pressure), het speltoid, strain  $\gamma$ 2. The C and C-deficient ("Cd") chromosomes are unassociated.

FIG. 36. Anaphase I, het speltoid, strain  $\gamma$ 1. The unassociated C and Cd have "split" after the bivalents have disjoined and their constituent chromatids are moving belatedly towards the poles.

FIG. 37. Metaphase I, het speltoid, strain  $\gamma$ 2. C and Cd are associated forming a heteromorphic bivalent (CCd $_{II}$ ).

FIG. 38. Pachytene, het speltoid, strain  $\gamma$ 2, interstitial deficiency demonstrated by unequal length of paired strands, presumably C and Cd, in a loop projecting from the main mass.

FIG. 39. Metaphase I, het speltoid, strain  $\alpha$  2, 20 $_{II}$  + CC $\alpha$  $_{II}$  (very slight heteromorphism).

FIG. 40. Anaphase I, anomalous bearded speltoid ex  $\gamma$ 2. A non-C trivalent has failed to be included in the daughter nuclei.

FIG. 41. Metaphase I showing heteromorphic bivalent from one head of a het speltoid-normal chimera.

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